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# RAPID AWARENESS AND TRANSMISSION OF SEVERE ACUTE RESPIRATORY SYNDROME IN HANOI FRENCH HOSPITAL, VIETNAM

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*Abstract.* A case-control study was conducted to examine the relationship between severe acute respiratory syndrome (SARS) and the time-dependent precautionary behaviors taken during an outbreak of SARS in Hanoi French Hospital (HFH), Vietnam. Masks (odds ratio [OR] = 0.3; 95% confidence interval [CI]: 0.1, 0.7) and gowns (OR = 0.2; 95% CI: 0.0, 0.8) appeared to prevent SARS transmission. The proportion of doctors and nurses who undertook each measure significantly improved ($\chi^2$ = 9.8551, $P$ = 0.043) after the onset of secondary cases. The impact of individual behaviors on an outbreak was investigated through mathematical approaches. The reproduction number decreased from 4.1 to 0.7 after notification. The basic reproduction number was estimated, and the use of masks alone was shown to be insufficient in containing an epidemic. Intuitive results obtained by means of stochastic individual-based simulations showed that rapid improvements in behavior and isolation would increase the probability of extinction.

## INTRODUCTION

Notwithstanding the announcement of containment by the World Health Organization (WHO) in 2003,[1] severe acute respiratory syndrome (SARS) has remained a matter of concern worldwide, and it is not surprising that several cases of SARS have reemerged, for example, in China in April 2004.[2] Although the mode of transmission remains partially unclear, especially with regard to airborne transmission[3] and super-spreading events,[4,5] it appears to occur predominantly by large droplets, direct contact with infectious material, or contact with fomites contaminated with infectious material.[6,7] The most effective containment measures identified to date include the tracing of contacts,[8] quarantine,[9] triage and early case detection,[10,11] and isolation.[12] Further, because the close contact required for transmission easily occurs in hospital settings,[13–15] nosocomial spread was determined as one of the major epidemiologic features of SARS.[7,16,17] The elimination of hospital transmission through enhanced infection control practices is therefore a crucial control measure.

An early study in Hong Kong showed that the practice of droplet and contact precautions was adequate in most clinical settings in significantly reducing the risk of infection after exposure to patients with SARS,[18] and if practiced by a high proportion of susceptible individuals, precautionary measures are expected to significantly reduce transmission.[19] The adoption of routine preventive behaviors based on appropriate training and control among health care workers (HCWs), undertaken prior to the isolation of SARS patients, was shown to be one of the most crucial control measures.[20–22]

In this context, Vietnam is considered to have achieved the first highly successful containment of SARS during the early phase of the outbreak.[23] One reason for this rapid containment is thought to be the prevention of infection leakage from hospitals back into the general community.[24] A second is the successful discontinuation of the chain of nosocomial transmission several days after onset based on the radical control measures of the Ministry of Health, Vietnam.[25] Although several nosocomial transmissions were observed in Hanoi French Hospital (HFH) in the early days of the outbreak,[26,27] none were identified in HFH or another local hospital in the latter phase.[28] In both hospitals, staff instituted stringent precautions, strict isolations, and quarantines under the encouragement of Dr. Carlo Urbani (Dr. Urbani died of SARS before seeing the success of the containment).[29] We therefore consider that a comprehensive understanding of the successful containment measures adopted by HFH and their theoretical underpinnings are crucial to the success of control strategies for any future recurrence. Here, we use a case-control study design to time-dependently examine the relationship between SARS and the precautionary behaviors undertaken by those exposed in HFH. We then use mathematical approaches to develop intuitive analyses of the impact of individual behaviors on the control of a SARS epidemic.

## MATERIALS AND METHODS

**Case-control study.** HFH is a 56-bed secondary care hospital. After the admission of an index case on February 26, 2003, 38 cases in total were confirmed to have symptomatic SARS infection. The occurrence of newly diagnosed SARS cases due to local transmission continued until April 7, 2003, 3 weeks before the date when the Vietnamese government and WHO declared the outbreak successfully contained (April 28, 2003) (Table 1). The duration of the HFH outbreak was analyzed by separating it into three phases: Stage 1, February 26–March 4, from admission of the index case to the onset of secondary cases; Stage 2, March 5–March 10, from the suspicion of nosocomial spread to closure of the hospital; and Stage 3, from March 11 on, from strict isolation to local eradication.

A case-control study of 29 of the 38 laboratory-confirmed SARS cases and 98 controls was performed in HFH. The case group included 22 of 28 (78.6%) individuals admitted and retained in HFH and 7 of 10 (70.0%) individuals transferred to another hospital after first being admitted to HFH (total $N$ = 29). The reasons for nonparticipation were death due to

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TABLE 1
Chronology of the outbreak of SARS in Hanoi French Hospital (HFH), Vietnam

| | | |
|---|---|---|
| Stage 1 | | |
| 26-Feb-03 | Day 0* | An index case complaining of fever, dry cough, and headaches was admitted to HFH. |
| 2-Mar-03 | Day 3 | After intubation, the index case was isolated in ICU the following day. |
| 4-Mar-03 | Day 6 | Nine secondary cases were suspected. |
| Stage 2 | | |
| 5-Mar-03 | Day 7 | Seven additional cases were suspected.<br>HFH informed the Ministry of Health, Vietnam, of the strange influenza.<br>The health minister and experts from the World Health Organization (WHO) held a meeting.<br>Dr. Carlo Urbani informed all staff to perform stringent precautions. |
| 8-Mar-03 | Day 10 | HFH decided to close all outpatient/inpatient services.<br>Visitors were not allowed to enter HFH.<br>The hospital board of directors held an emergency meeting.<br>Dr. Carlo Urbani explained the necessity of precautions and possibility of contamination as a mode of transmission.<br>Health care workers were advised not to return home. |
| Stage 3 | | |
| 11-Mar-03 | Day 13 | All inpatients were transferred to other hospitals.<br>The 2nd floor of HFH was allocated to SARS patients only and strict isolation was enforced.<br>• Three zones were allocated according to symptoms.<br>• Nonmission individuals including health care workers were not allowed to enter. |
| 13-Mar-03 | Day 15 | A special committee for SARS control and prevention was established.<br>WHO issued a "global alert" to worldwide health authorities. |
| 28-Apr-03 | Day 60 | The Vietnamese government and WHO declared successful containment of SARS in Vietnam. |

* Day, days after onset of the outbreak. SARS, severe acute respiratory syndrome; ICU, intensive care unit.

SARS and/or respiratory failure ($N$ = 5, 13.2%), refusal to take part ($N$ = 1, 2.6%), or relocation ($N$ = 3, 7.9%). The case group included 28 HFH employees (3 doctors, 13 nurses and nursing assistants, 10 radiologists and other co-medical workers, and 2 receptionist and administrative staff) and 1 relative of a patient. A further 23 Vietnamese patients who were directly admitted to another hospital were excluded because the detailed source of infection was unknown, although several cases were thought to have been infected in HFH. Detailed descriptions of the laboratory diagnoses were given previously.[28] They were confirmed through serological studies using an indirect enzyme-linked immunosorbent assay (ELISA) (Kirikae T, et al., unpublished data).

Controls were nominated based on employment in HFH and exposure among patients' relatives through HFH. The selection criteria included i) Vietnamese individuals more than 20 years old, ii) those who provided written informed consent based on explanation of our methods and purposes, and iii) those thought to have had contact with confirmed cases inside the hospital based on contact investigations. In total, 98 individuals were included as controls; most were HFH employees (13 doctors, 20 nurses and nursing assistants, 13 radiologists and other co-medical workers, and 11 receptionists and administrative staffs) or relatives of patients ($N$ = 41). Although we investigated certain known contacts for inclusion as controls, namely individuals who took care of cases or entered cases' room, those who might have had trivial contact, such as possible exposure outside the hospital during, for example, transportation of SARS cases or in the casualty reception room, were not followed and included. The number of hospital employees investigated represented approximately 55.9% of the total employees used during the outbreak.

All participants were surveyed with regards to their use of personal protective equipment (PPE) and hygiene habits when in contact with patients with SARS; that is, the use of masks, gloves, and gowns, and the practice of hand washing, which were specifically recommended as droplet and contact precautions. In this paper, masks denote surgical masks; N95 masks were not available in the early stage of the outbreak in Vietnam. Individual behaviors were investigated mainly in two separate phases according to time-dependency (in Stage 1 and after entering Stage 2; i.e., Stages 2 and 3) (Table 1) to clarify any behavioral changes that occurred. Standardized questionnaires requiring one of two possible answers for each precaution ("performed" or "not performed") were given to each subject, and all responses were collected. Answers of "sometimes" or "seldom" were defined as "not performed" due to imperfect efficacy. In addition, the frequency of contact with infected individuals was investigated to represent the number of exposures per day. An exposure result of "many times" was recorded for those who had close contact with SARS patients, that is, those who cared for or lived with SARS patients, and those likely to have come into direct contact with the respiratory secretions or body fluids of SARS patients, for example, during close conversation (within 3 feet).[30] After completing the initial primary survey, an identical confirmation survey was performed to confirm the validity of the answers. These surveys were conducted along with other epidemiologic studies (Nishiyama A, et al., unpublished data) until mid-March 2004, almost 1 year after onset of the epidemic. No blood test results showing possible asymptomatic infections were available during the survey period. The participants were informed of how the information would be used and assured of the confidentiality of their responses. The purpose of the study was explained in Vietnamese, and written informed consent was obtained.

Statistical analyses were performed as follows. First, univariate associations between precautionary behaviors and infection were investigated in two separate stages (Stage 1 or Stages 2 and 3). Comparisons between groups were made using the $\chi^2$ or Fisher's exact test for univariate analysis. Multivariate logistic regression was done in Stage 1 using forward stepwise selection (Waldesian) to determine the most significant variable associated with protection among those studied. Significant steps were taken to minimize recall bias with Stages 2 and 3 data. Analysis was restricted to those who had probable contact in these stages. It was further restricted to those cases developing symptoms whose incubation period

was within the greater than 95% confidence interval (95% CI) of having occurred after the beginning of Stage 2; and finally to medical doctors and nurses only, for both cases and controls. Second, univariate associations between sociodemographic variables (sex, age, and occupation) and SARS were investigated, with age and occupation categorized into four different groups each. Third, interactions between the identified most significant protective behavior and other variables significantly associated in univariate analysis were investigated through the use of crosstabs statistics, in which the odds of being infected were stratified according to a comparison of variables, and interactions were sought through the different odds ratio in each strata. Finally, multiple logistic regression analysis was used to determine the protective effect and eliminate confounding variables. As described in the next section, all variables significantly associated in univariate analyses, as well as sociodemographic variables, were selected and entered together in the final model. All data were entered into Microsoft Excel 2000 (Microsoft Co., Redmond, WA), and the statistical data were analyzed using the statistical software "R" (R Development Core Team, Vienna).[31]

**Mathematical methods.** The predictive effects of the behavioral changes were simulated using an individual-based stochastic model. For ease of understanding, a compartmental model, a type of SEIR (susceptible [$S$], exposed [$E$], infected [$I$], and recovered/removed [$R$]) model, which considered the process of transmission according to the protective behaviors taken against infectious contact among susceptible individuals, was applied. Instead of assuming "exposed (latent)" and "infectious" periods, $E$ and $I$ were defined as "incubation" and "symptomatic" periods, respectively, as the infectious period of SARS has not been fully clarified. Although SEIR models are usually deterministic and use mean estimations as model parameters, even with regard to SARS,[12,32] stochastic simulations were performed in this study because of the need to consider the stochasticity of each protective behavior, and also because of the small sample population size. The infectious lifetime of each individual was presented as an absorbing Markov chain. The simulations start with an individual index case (Day 0) in a population of 300 in which all individuals are susceptible.

Of the total 127 subjects studied (29 cases and 98 controls), 62.2% ($N = 79$) were considered to have had casual contact and 37.8% ($N = 48$) to have had close contact with SARS patients. The number of casual contacts ($\kappa_1$) was directly obtained ($= 0.7 \pm 0.2$ [day$^{-1}$]), while the mean of close contacts ($\kappa_2 = 0.4$ [day$^{-1}$]) was determined with the following equation:

$$\kappa_2 = \kappa_1 \ln(\mathrm{OR}_{\mathrm{closed}}) \quad (1)$$

where $\mathrm{OR}_{\mathrm{closed}}$ (= 2.5; 95% CI: 1.1–5.9) denotes the odds ratio (OR) of getting infected as a result of close contact. In other words, to quantify close contact, we assumed that the frequency of infection is mainly determined by the frequency of contact, so that the ratio of the frequency of close to casual contact becomes proportional to the logarithm of the OR of transmission. The protective effect of precautionary behavior was approximated by:

$$\beta = 1 - \mathrm{RR} = 1 - \frac{a(c+d)}{c(a+b)} \approx 1 - \frac{ad}{bc} \approx 1 - \mathrm{OR} \quad (2)$$

where RR and OR denote the relative risk and odds ratio, respectively, of becoming infected while performing a protective behavior (with precaution = with exposure). Here, $a$ is the number of exposed ill people; $b$, the number of exposed healthy people; $c$, the number of unexposed ill people; and $d$, the number of unexposed healthy people. If the outcome (i.e., disease investigated) is a rare event, that is, if $a$ and $c$ are very small compared with $b$ and $d$, respectively, $(a + b)$ and $(c + d)$, respectively, would be closely similar to $b$ and $d$ alone. In this case, OR would approximate RR.

The lengths of the incubation and symptomatic periods were both assumed to be independently and identically distributed random variables with a probability density function of $\gamma$ distribution, the mean and variance of which were defined as 3.8 [days] and 8.3 [days$^2$], and 16.2 [days] and 7.9 [days$^2$], respectively.[24,33] These distributions were applied to difference equations (as a discrete time model) by discretizing the probability density functions by day (for a detailed description of the simulation algorithm, see the Appendix).

The first simulation scenario hypothetically investigated the unchanged coverage and mean protective effects of a behavioral measure throughout the epidemic. Primary information on protective behaviors was obtained from our Stage 1 survey. Estimates for the extent of a protective effect, the associated causative behavior of which was found in forward stepwise logistic regression to be the most significantly associated with protection (as described above), were obtained through the use of further multivariate logistic regression analysis. This analysis incorporated all variables significantly associated with SARS on univariate analysis (i.e., other precautionary behavior, gender, age and occupation). To investigate the impact of the coverage of a protective measure on the trajectory of an outbreak, sensitivity of the cumulative number of SARS cases at Day 30 to the coverage of masks was investigated in the mean field equation. In the second scenario, it was assumed that coverage improved dramatically after entering Stage 2 (Day 7) due to an awareness of transmission. Further, in Stage 3 (Day 13), the hospital implemented not only stringent precautions but also strict isolations. To understand the trajectory of transmission in detail, the number of incubating as well as symptomatic individuals was investigated. As was in fact seen during Stage 3 of the outbreak, it was also assumed that all cases who became symptomatic were immediately isolated and that nobody except a limited number of healthcare workers were permitted to have contact with them. Because the greatest uncertainty applies to the time taken to increase coverage of a protective measure and to implement strict isolations, sensitivity analyses comparing the cumulative number of SARS cases up to Day 30 were performed with the time to change both protective measures set simultaneously on the same day. Finally, the basic reproduction number was estimated using the (effective) reproduction number obtained in Stage 1 (see Appendix).

## RESULTS

Table 2 shows the univariate association between the precautionary behaviors taken (SARS and non-SARS [control] cases) in Stage 1 and SARS. The use of masks ($P = 0.011$) and gowns ($P = 0.012$) appeared to prevent infection, whereas handwashing and the use of gloves were less likely to provide protection. Only two subjects who performed all pro-

TABLE 2
Precautionary measures taken by all participants in Stage 1

| | SARS cases ($N$ = 25) | Non-SARS ($N$ = 90) | $P$ value* | Odds ratio† (95% CI)‡ |
|---|---|---|---|---|
| All measures | 2 | 44 | 0.059 | 0.2 (0.0–1.0) |
| Handwashing before§ | 12 | 51 | 0.937 | 1.0 (0.4–2.3) |
| Handwashing after¶ | 15 | 56 | 0.766 | 1.1 (0.5–2.8) |
| Masks‖ | 8 | 35 | 0.011 | 0.3 (0.1–0.7) |
| Gloves | 8 | 30 | 0.643 | 0.7 (0.3–1.9) |
| Gowns | 2 | 25 | 0.012 | 0.2 (0.0–0.8) |

* Two-tailed.
† Odds ratio of being infected while taking specific precautions.
‡ 95% CI: 95% confidence interval.
§ Hands washed before having contact with a patient.
¶ Hands washed after having contact with a patient.
‖ Only those who always used a mask.

tective measures developed symptomatic infections ($P$ = 0.059). Forward stepwise logistic regression of the five protective measures (0.05 for entry and 0.10 for removal probability) showed that only the use of masks was significant in the final model (OR, 0.29, 95% CI; 0.11-0.73, $P$ = 0.009). In Stages 2 and 3, the use of masks ($P$ = 0.001) and gowns ($P$ = 0.010) was significantly associated with non-infection among doctors and nurses still not infected after Stage 1 (Table 3). Most performed all the personal protective measures recommended, and only one individual who wore masks was infected. The comparative results of the behaviors of all participants at Stage 1 and after entering Stage 2 are shown in Figure 1a. The proportions of individuals who performed the investigated protective behaviors increased after entering Stage 2. However, these behavioral changes were not significantly different between the two phases ($P$ = 0.960). The behaviors performed by the doctors and nurses ($N$ = 48; Figure 1b) who had the closest contact with the SARS patients drastically and significantly improved after entering Stage 2 ($\chi^2$ = 9.855, $P$ = 0.043).

The univariate associations between socio-demographic variables and SARS throughout the epidemic are shown in Table 4. Females were more likely to become infected than males ($P$ = 0.011), and a significant association of SARS with nurses ($P$ = 0.008) was observed. In HFH, infection was frequent in the 40–49 age strata ($P$ = 0.015). Among all study subject, relatives of patients ($P$ < 0.001) appeared to be the least frequently infected. Table 5 shows the interaction between the use of masks and other significantly associated variables in univariate analyses. Even though we saw no significant difference in the OR of using masks versus the use of gowns, females (OR = 0.2) and nurses (OR = 0.1) were more effectively protected by the use of masks than others in Stage 1. In Stages 2 and 3, the use of gowns showed overall reasonable OR (= 0.2), whereas most other interactions could not be calculated due to the scarcity of cases.

Figure 2a shows the mean and corresponding 95% CI of the trajectory (shown as prevalence) of an epidemic from 250 simulation runs which hypothetically assumed unchanged coverage as well as the protective effects of the precautionary measures observed in Stage 1. The precautionary measure in this simulation was based on a multivariate logistic regression which included all variables showing significant associations in univariate analyses, and focused on the impact of the use of masks, given the identification of this behavior as the most important protective measure ($\beta$ = 0.6 obtained from OR = 0.4, $P$ = 0.020). The coverage of masks was obtained as 52.0% from Table 2. If an outbreak was simply allowed to continue growing under these conditions, the results showed that approximately 50 to 90 symptomatic cases would occur by Day 30. The reproduction number ($R$) was estimated as 4.1 (95% CI; 1.9–6.4), and from this estimate the basic reproduction number was estimated as 6.0. Sensitivity of the cumulative number of cases to the coverage of masks, in the mean field, is shown in Figure 2b. Certain reduction in the cumulative number of cases was observed with significant improvements in coverage.

Figures 2c and 2d shows the outbreak trajectory of 250 simulations assuming improved coverage (from 52.0 to 81.5%) among susceptible individuals on Day 8 and restriction of contact with symptomatic individuals to health care workers on Day 13. The protective effect obtained from multivariage regression was 0.9 (OR = 0.1, $P$ = 0.955). The reproduction number in Stage 2 was estimated as 0.7 (95% CI; 0.0–2.3). The number of incubating individuals began to show a decreasing trend after these events (Figure 2c), followed by a declining trend in the number of symptomatic cases (Figure 2d). Most of the simulated outbreaks eventually declined to extinction before Day 120. The sensitivity of the final size of an epidemic, evaluated through observations of the cumulative numbers of cases, to the timing of drastic changes in protective behaviors accompanied by strict isolation is shown in Figure 2e. When the stochastic effects are taken into account together with the effects of single precautionary measures and isolation, the rapid implementation of combined measures reduces the number of transmissions and increases the probability of extinction.

TABLE 3
Precautionary measures taken by health care workers in Stages 2 and 3

| | SARS cases ($N$ = 4) | Non-SARS ($N$ = 26) | $P$ value* | Odds ratio† (95% CI)‡ |
|---|---|---|---|---|
| All measures | 1 | 25 | 0.001 | < 0.1 (0.0–0.3) |
| Handwashing before§ | 4 | 25 | 1.000 | NC |
| Handwashing after¶ | 4 | 25 | 1.000 | NC |
| Masks‖ | 1 | 25 | 0.001 | < 0.1 (0.0–0.3) |
| Gloves | 4 | 25 | 1.000 | NC |
| Gowns | 3 | 26 | 0.010 | NC |

* Two-tailed.
† Odds ratio of being infected while taking specific precautions.
‡ 95% CI: 95% confidence interval.
§ Hands washed before having contact with a patient.
¶ Hands washed after having contact with a patient.
‖ Only those who always used a mask.

## DISCUSSION

The findings of this case-control study indicate that the use of masks was significantly associated with the prevention of SARS transmission and that precautions against droplet contamination and contact were adequate in preventing transmission; this implies mainly to in-hospitals. The results are roughly consistent with those of previous reports.[18,20,22] Although a number of exceptions were seen with regard to protective effects during patient intubation, during which transmission to staff occurred even when droplet and contact precautions were taken,[7,34] one of the most important lessons from the SARS outbreak is the need to enhance infection control programs in hospitals.[13,35] Even though the use of

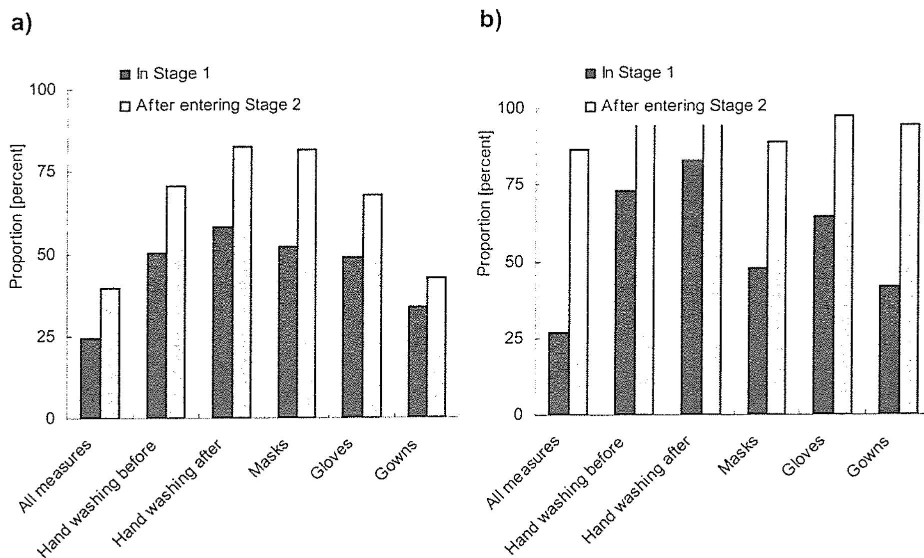


FIGURE 1. Protective behavioral changes defined by stage. **a**, Proportion of participants (SARS and non-SARS [control] cases) who performed each precautionary measure in Stage 1 ($N$ = 127) and after entering Stage 2 ($N$ = 108). Handwashing "before" and "after" denote before and after contact with a patient, respectively. **b**, Proportion of health care workers who performed each precautionary measure in Stage 1 ($N$ = 48) and after entering Stage 2 ($N$ = 37).

masks was the most effective precautionary measure, masks alone together with the observed coverage did not reduce the reproduction number below unity ($R_0$ = 6.0 and $R$ with the protective effects of masks = 4.1). Put simply, the use of masks alone was shown to be insufficient to contain the epidemic. Further, it was shown that the coverage of precautionary behaviors among the study subjects increased with the progression of the outbreak, and this was especially obvious among doctors and nurses. In HFH, remarkable changes occurred in the very early phase of the outbreak before detailed information about SARS was available. According to the stochastic simulations, an increased probability of extinction would be observed if the combined measures of precaution and isolation were rapidly implemented.

With regard to sociodemographic variables, females were more frequently infected than males. Given that transmission was most frequently observed among nurses, a plausible explanation for this finding would be occupational background. Although the 40–49 age group was frequently infected, we have no persuasive explanation for this apart from occupation: 61.9% of this stratum was medical doctors or nurses. Considering that nurses were more effectively protected from transmission by the use of masks, the control measures taken by them within HFH from early in the epidemic were admirable. The lowest frequency of infection was seen in relatives of patients, showing that our study included many relatives who remained uninfected but were nevertheless believed to have had contact. Because nonmatched case-control designs such as this are vulnerable to selection bias, we obtained estimates of the protective effect of masks by means of multivariate logistic regression analysis which entered all other variables significantly associated with infection in univariate analysis. After adjustment for internal confounding variables, the estimated reproduction number was given as 0.7 in Stages 2 and 3. Previous studies have shown that the (effective) reproduction number, defined as the average number of secondary cases generated by one index case in a susceptible population under certain restrictions and interventions, decreases with increasing awareness of the epidemic combined with several public health measures.[36,37] Using reasonable estimation procedures, another study showed that $R$ significantly decreased after a global alert in most affected countries.[38] The current study showed that the estimated $R$ decreased below unity after notification of a hospital outbreak, although the estimates were obtained using rough assumptions and the process of estimation was biased by various factors.

In HFH, the rapid increase in awareness, which led to not only strengthened precautionary measures and isolation but also quarantining of health care workers, seems to have been the greatest contributor to successful containment. One reason for this quick response could be attributed to the background of secondary cases that arose mainly from health care workers who had close contact with the index case. Almost all staff members working or on duty in the earliest days of the

TABLE 4
Univariate associations between age-class/occupational categories and SARS

| Category | | $N$ | $P$ value* | Odds ratio (95% CI)† |
|---|---|---|---|---|
| Sex | Male | 47 | 0.011 | 0.3 (0.1–0.8) |
| | Female | 70 | 0.011 | 3.3 (1.2–9.0) |
| Age class | 29 y/o | 29 | 1.000 | 0.9 (0.3–2.3) |
| | 30–39 y/o | 44 | 0.080 | 0.4 (0.2–1.1) |
| | 40–49 y/o | 42 | 0.015 | 2.8 (1.2–6.6) |
| | 50 y/o | 12 | 0.733 | 0.7 (0.1–3.2) |
| Occupation | Medical doctors | 16 | 1.000 | 0.8 (0.2–2.9) |
| | Nurses | 33 | 0.008 | 3.2 (1.3–7.7) |
| | Other co-medicals | 36 | 0.076 | 2.2 (0.9–5.2) |
| | Relatives of patients | 42 | < 0.001 | < 0.1 (0.0–0.4) |

* Two-tailed.
† Odds ratio of being infected while taking specific precautions.

TABLE 5
Interactions between wearing masks and other variables on the infection

| | In stage 1 | | | In stages 2 and 3 | | |
|---|---|---|---|---|---|---|
| | Odds for masks (+) | Odds for masks (−) | Odds ratio* | Odds for masks (+) | Odds for masks (−) | Odds ratio* |
| Gowns | | | | | | |
| (+) | 0.3 | 0.6 | 0.5 | < 0.1 | 2.0 | 0.2 |
| (−) | 0.3 | 0.5 | 0.6 | NC | NC | NC |
| Sex | | | | | | |
| (male) | 0.1 | 0.2 | 1.0 | 0.0 | 0.0 | NC |
| (female) | 0.2 | 0.8 | 0.2 | 0.1 | NC | NC |
| Age class | | | | | | |
| 29 y/o | 0.1 | 0.4 | 0.3 | 0.0 | NC | NC |
| 30–39 y/o | 0.1 | 0.3 | 0.5 | 0.0 | NC | NC |
| 40–49 y/o | 0.3 | 0.8 | 0.3 | 0.1 | 1.0 | 0.1 |
| 50 y/o | 0.2 | 0.2 | 1.0 | 0.0 | NC | NC |
| Occupation | | | | | | |
| (Medical doctors) | NC | 0.6 | NC | 0.0 | 0.0 | NC |
| (Nurses) | 0.2 | 1.6 | 0.1 | 0.1 | 0.0 | NC |
| (Other co-medicals) | 0.5 | 0.5 | 1.2 | | | |
| (Relatives of patients) | NC | 0.1 | NC | | | |

NC = not calculable.
* Odds ratio of being infected while taking specific precautions.

outbreak (in Stage 1) were severely infected.[39,40] Another reason might be due to the efforts led mainly by Dr. Carlo Urbani, who suggested quick improvements in the precautionary measures taken and isolation.[29] As a result, transmission leakage into the community was prevented, thus having a huge impact on the chains of transmission.[24] In HFH, those who were exposed implemented precautionary and other controlling measures quickly and efficiently, and the epidemic consequently declined to extinction.

In the interests of objective interpretation, the limitations of our study design must be addressed, as follows:

1) A study such as ours in which exposure has a strong intuitive causal link with outcome (i.e., mask usage) is vulnerable to recall bias. Even though we limited our subjects in Stages 2 and 3 to medical doctors and nurses, and cases were appropriately selected according to the probable date of infection and incubation period, our estimates are likely less accurate than would be obtained by blinded or matched case-control study. In addition to this directional bias, further bias may have been introduced by random misclassification, as our records were completed 1 year after the outbreak, and it is therefore possible that some of the precautions were uncertain exposures. The frequent use of masks among controls may have reduced the strength of the associations.
2) Model-generated results must be interpreted cautiously. Although the simulations shown here included only the effect of masks and were considered according to the results of multivariate logistic regression adjusted for internal factors, unknown external confounding factors likely exist. For example, in Stages 2 and 3, although multivariate logistic regression was performed with other variables, the $P$ value obtained was 0.955, and overall the model was weak. Owing to the scarcity of case records, stratification in this stage failed to separate the effects of masks. Thus, the estimates of the protective effect of masks and reproduction number in this stage may include the effects of other concomitant changes, such as the reduced frequency of contacts and quarantine.
3) There are limitations concerning the simplicity of our model; for example, we neglected the possible differential susceptibility of humans to asymptomatic infections,[41,42] individual variance in severity and/or prognosis,[23,43,44] and the highly heterogenous transmission of SARS.[4,5,45] Theoretical exercises never replace reality.
4) Finally, because our model was based on a case-control study, the estimates of coverage were biased; principally, coverage in a case-control design is taken from a nonrepresentative sample. Although this study was conducted as a first attempt to incorporate the effect of behavioral factors, which change time-dependently, to model building strategies for the control of directly transmitted airborne diseases, further studies incorporating a number of methodological improvements are required.

In conclusion, given that early recognition that leads to the implementation of protective behaviors and effective control strategies is crucial in hospitals,[46] we believe our model provides intuitive results that at least partly satisfy the need to evaluate outbreak trajectories based on individual behaviors.

## APPENDIX

Each simulation starts with one index case and is based on a model constructed as follows:

i) The expected number of people who used protection on each subsequent day was determined by the number of susceptible individuals ($S$), number of contacts per day ($\kappa$), proportion of individuals who performed the protective behavior ($p$), and the protective effect of the precautionary measure ($\beta$), which were obtained based on our survey. The number of infectious contacts, denoted by the product of the number of susceptible individuals ($S$) and the mean number of contacts ($\kappa$), was divided into two subgroups: one that represents protection due to precautionary behaviors against infection with SARS-CoV (SARS-associated coronavirus) and another that does

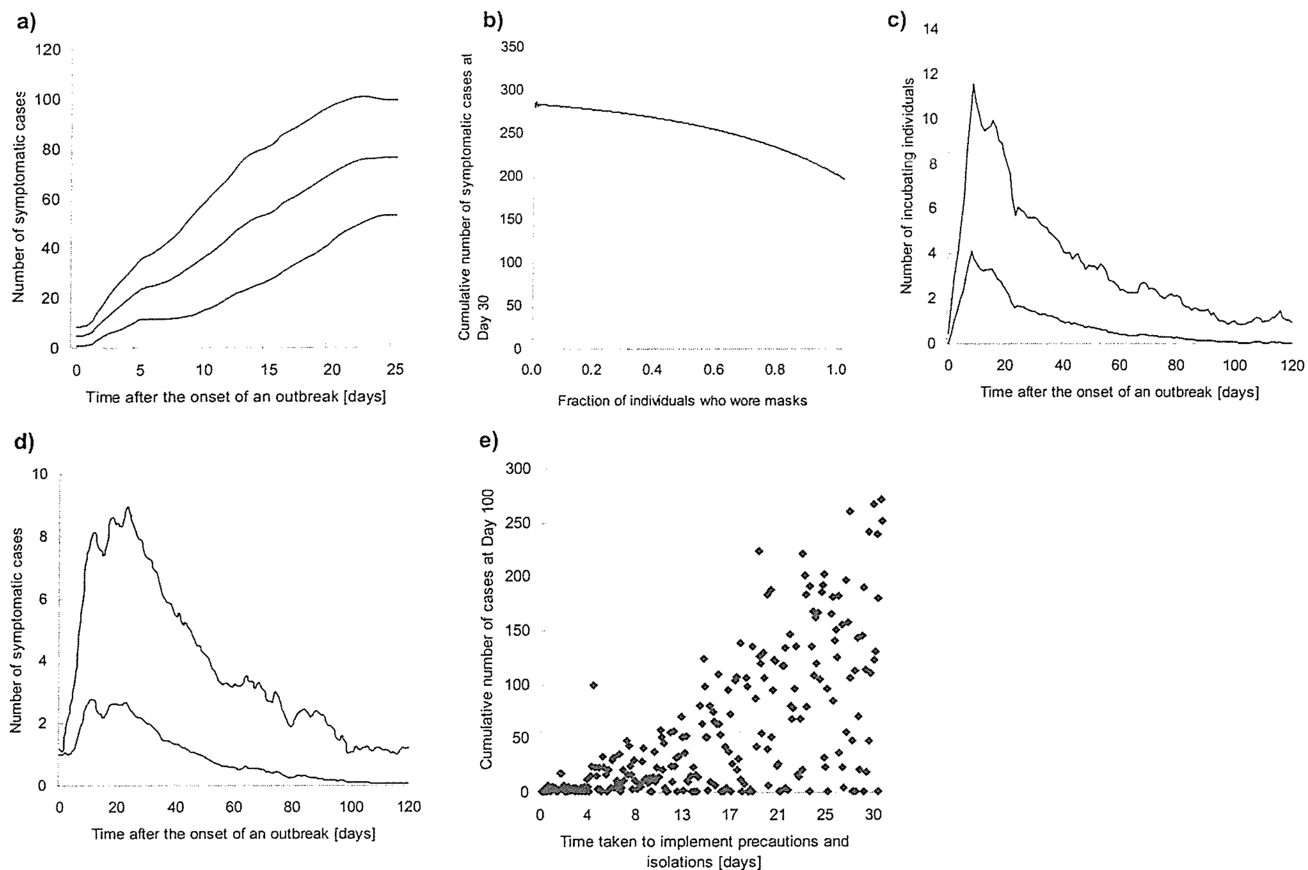


FIGURE 2. Stochastic simulations of a SARS outbreak with dependency on the coverage and protective effect of precautionary behaviors. **a**, Predicted number of symptomatic cases and corresponding 95% confidence interval (95% CI) given by 250 simulation runs assuming unchanged (stable) protective behaviors for the entire period. The reproduction number ($R$) was 4.1 ± 1.1. **b**, Sensitivity of the cumulative number of cases at Day 30 to the coverage of masks. The obtained line represents the simulation based on mean field (without assuming random function with binomial distribution in each transition probability). The protective effect of wearing a mask was fixed ($\beta = 0.6$). **c** and **d**, Stochastic simulations of a SARS outbreak with dependency on a combination of precautionary measures and strict isolation. **c**, The mean number of incubating individuals and corresponding 95% CI from 250 runs with changes in protective behaviors combined with strict isolation (lower 95% CI is $x$-axis). At Day 7, the effectiveness/coverage of precautionary measures used improved from 0.6/52.0 to 0.9/89.2, respectively. At Day 13, the number of susceptible individuals decreased from 300 to 20. The reproduction number decreased from 4.1–0.7 ± 1.1–0.8. **d**, The mean ± 95% CI of symptomatic cases given by 250 runs assuming changes in protective behaviors combined with strict isolation. The conditions were the same as those in **c**. **e**, Sensitivity of the size of an outbreak (represented by the cumulative number of cases) to the time taken to enhance precautionary measures and implement strict isolation; the combined measures are started at the same time and under the same conditions as in **c**.

not, according to $(1 - p\beta)$. However, these groups were not permanently fixed. The mean of the number of contacts based on our survey was approximated by:

$$\kappa = \kappa_1\pi_1 + \kappa_2\pi_2 = \kappa_1\pi_1 + \kappa_1 \ln(\mathrm{OR}_{close})\pi_2 \qquad \text{(A1)}$$

where $\kappa_1$, $\kappa_2$, $\pi_1$, and $\pi_2$ denote the number of casual and close contacts and the fraction of individuals who had casual and close contacts, respectively, while the odds ratio of getting infected with close contact is represented by $\mathrm{OR}_{close}$ and $N$, respectively.

ii) Both the incubation ($E$) and symptomatic ($I$) periods were assumed to be independently and identically distributed following an approximated probability density function with gamma distributions[33] (denoted by $\gamma_k$ and $c_l$ for the discretized stages [days] $k$ and $l$, respectively). We divided the probability density functions into $k$ ($i$ = 14) and $l$ ($j$ = 12) stages; the methodology of approximation by date was previously reported.[24] The relative measure of infectiousness for the incubation ($E$) period ($q$) was assumed to be 0.1.[12]

iii) Based on realistic settings in Vietnam, it was assumed that all individuals were isolated with the onset of early signs of clinical symptoms under the isolation measures; and for simplicity, the effect of quarantine was neglected. When considering strict isolation, the number of susceptible individuals having contact with SARS patients was limited to 20 (which is the approximate number of ward workers); the number of susceptible individuals was treated as being stable (always $S$ = 20) so that $S$ would not be exhausted thereafter; without isolation there were assumed to be 300 susceptible individuals (which is roughly the total number of people involved in possible contacts in HFH). $N = S + E + I + R$, and background mortality was neglected. The resulting simplest difference equations were formulated as follows:

$$
\begin{aligned}
S(t+1) &= \exp\left[-\kappa(1-p\beta)\frac{I+qE}{N}\right]S(t) \\
E_1(t+1) &= \left\{1-\exp\left[-\kappa(1-p\beta)\frac{I+qE}{N}\right]\right\}S(t) \\
E_k(t+1) &= (1-\gamma_{k-1})E_{k-1}(t) \qquad \text{(A2)} \\
I_1(t+1) &= \sum_{k=1}^{i}\gamma_k E_k(t) \\
I_l(t+1) &= (1-c_{l-1})I_{l-1}(t) \\
R(t+1) &= R(t) + \sum_{l=1}^{j} c_l I_l(t)
\end{aligned}
$$

Based on the forward stepwise logistic regression result in the case-control study, and to facilitate understanding, $p$ and $\beta$ were used only to represent the use of masks. However, the protective effect, $\beta$, was obtained from the result of further multiple logistic regression which entered all other significantly associated variables (in univariate analysis). All terms shown here as products of a probability and a state variable were generated in our simulations by using random variables with binomial distributions. Under these assumptions and using mean length of incubation and symptomatic periods, the reproduction number ($R$) is given by:

$$R = \kappa(1-p\beta)\left(\frac{q}{\gamma}+\frac{1}{c}\right) \qquad \text{(A3)}$$

where $\gamma^{-1}$ and $c^{-1}$ are the means of the incubation and symptomatic periods in days, respectively. The basic reproduction number was estimated by

$$R_0 = \frac{R}{(1-p\beta)} \qquad \text{(A4)}$$

For the purpose of mathematical convenience, although unrealistic, our model assumed homogenous mixing as well as all infectious individuals being equally infectious.

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# Multidrug-Resistant *Pseudomonas aeruginosa* Strain That Caused an Outbreak in a Neurosurgery Ward and Its *aac(6')-Iae* Gene Cassette Encoding a Novel Aminoglycoside Acetyltransferase

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**We characterized multidrug-resistant *Pseudomonas aeruginosa* strains isolated from patients involved in an outbreak of catheter-associated urinary tract infections that occurred in a neurosurgery ward of a hospital in Sendai, Japan. Pulsed-field gel electrophoresis of SpeI-, XbaI-, or HpaI-digested genomic DNAs from the isolates revealed that clonal expansion of a *P. aeruginosa* strain designated IMCJ2.S1 had occurred in the ward. This strain possessed broad-spectrum resistance to aminoglycosides, β-lactams, fluoroquinolones, tetracyclines, sulfonamides, and chlorhexidine. Strain IMCJ2.S1 showed a level of resistance to some kinds of disinfectants similar to that of a control strain of *P. aeruginosa*, ATCC 27853. IMCJ2.S1 contained a novel class 1 integron, In113, in the chromosome but not on a plasmid. In113 contains an array of three gene cassettes of $bla_{IMP-1}$, a novel aminoglycoside resistance gene, and the *aadA1* gene. The aminoglycoside resistance gene, designated *aac(6')-Iae*, encoded a 183-amino-acid protein that shared 57.1% identity with AAC(6')-Iq. Recombinant AAC(6')-Iae protein showed aminoglycoside 6'-*N*-acetyltransferase activity by thin-layer chromatography. *Escherichia coli* expressing exogenous *aac(6')-Iae* showed resistance to amikacin, dibekacin, isepamicin, kanamycin, netilmicin, sisomicin, and tobramycin but not to arbekacin, gentamicins, or streptomycin. Alterations of *gyrA* and *parC* at the amino acid sequence level were detected in IMCJ2.S1, suggesting that such mutations confer the resistance to fluoroquinolones observed for this strain. These results indicate that *P. aeruginosa* IMCJ2.S1 has developed multidrug resistance by acquiring resistance determinants, including a novel member of the *aac(6')-I* family and mutations in drug resistance genes.**

*Pseudomonas aeruginosa* is intrinsically resistant to many antibiotics; however, it is sensitive to a limited number of drugs, including some β-lactams, such as ceftazidime and imipenem, and aminoglycosides, such as amikacin and tobramycin. However, recent studies have shown that several strains of *P. aeruginosa* that are resistant to these antibiotics have emerged and are becoming widespread (21, 28).

In Japan, the major mechanism of resistance to aminoglycosides is production of aminoglycoside-modifying enzymes (43). The aminoglycoside 6'-*N*-acetyltransferases [AAC(6')s] are of particular interest because they can modify a number of clinically important aminoglycosides including amikacin, gentamicin, netilmicin, and tobramycin. The AAC(6')-I type confers resistance to amikacin through acetylation of the drug, whereas the AAC(6')-II type acetylates gentamicin. To date, several different genes, designated *aac(6')-Ia* to *aac(6')-Iad*, that encode the AAC(6')-I enzymes have been cloned and characterized (42, 50). Genes encoding aminoglycoside-modifying enzymes are often located on integrons (15), sequences that can integrate gene cassettes through site-specific recombination (17), in both plasmid and genomic DNA (15). Class 1 integrons participate in multidrug resistance in *P. aeruginosa* (27, 28, 37). Class 1 integrons contain two conserved segments (CS) that flank the antibiotic resistance gene cassettes. The 5'-CS contains the *intI1* gene, which encodes integrase, the enzyme responsible for catalysis of site-specific recombination (8). The 3'-CS contains the *qacE*Δ*1* and *sul1* genes and an open reading frame (ORF), orf5 (13, 16).

We describe here the genotypic and phenotypic properties of a new multidrug-resistant *P. aeruginosa* strain that caused a nosocomial outbreak of infection at a hospital in Japan. The isolate carries a class 1 integron that contains an array of three gene cassettes, including one encoding a novel aminoglycoside acetyltransferase.

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## MATERIALS AND METHODS

**Bacterial strains.** Seven clinical isolates of *P. aeruginosa*, including *P. aeruginosa* IMCJ2.S1, were obtained from seven patients with urinary tract infections in a neurosurgery ward of a hospital in Japan. *P. aeruginosa* ATCC 27853 was obtained from the American Type Culture Collection (Manassas, Va). *Escherichia coli* strains DH5α (Takara Bio, Shiga, Japan) and BL21-AI (Invitrogen, Carlsbad, Calif.) were used as hosts for recombinant plasmids and for expression of *aac(6')-Iae*, respectively. The rifampin-resistant *P. aeruginosa* mutant ATCC 27853 $RFP^r$ was used. *P. aeruginosa* GN17203 (51) was provided by S. Iyobe (Kitasato University, Sagamihara, Japan).

**Antibiotics and disinfectants.** The antibiotics amikacin, cefoxitin, and imipenem were from Banyu Pharmaceutical Co. (Tokyo, Japan). Arbekacin and dibekacin were from Meiji Seika Kaisha (Tokyo, Japan), aztreonam was from Eizai (Tokyo, Japan), cefotaxime was from Aventis Pharma (Tokyo, Japan), and cefpodoxime and ceftazidime were from Glaxo Smith Kline (Tokyo, Japan). Cefepime was from Bristol Pharmaceuticals (Tokyo, Japan); ciprofloxacin and levofloxacin were from Daiichi Pharmaceutical (Tokyo, Japan); gentamicin, isepamicin, netilmicin, and sisomicin were from Schering-Plough (Osaka, Japan); kanamycin A and B mixture, neomycin B and C mixture, and streptomycin were from Nacalai Tesque (Kyoto, Japan); and meropenem was from Sumitomo Pharmaceutical (Osaka, Japan). Tetracycline was from Lederle Japan Co. (Tokyo, Japan); piperacillin and piperacillin-tazobactam were from Tomiyama Pure Chemical Industries (Tokyo, Japan); moxalactam, tobramycin, and sulfamethoxazole-trimethoprim were from Shionogi and Co. (Osaka, Japan); and kanamycin A, polymyxin B, and silver sulfadiazine were from Sigma Chemical (St. Louis, Mo.). The disinfectants alkyldiaminoethylglycine hydrochloride and povidone iodine were from Yoshida Pharmaceutical Co. (Tokyo, Japan); benzalkonium chloride was from Wako Pure Chemical Industries (Osaka, Japan); and chlorhexidine gluconate was from Ishimaru Pharmaceutical (Osaka, Japan).

**In vitro susceptibility to antibiotics and disinfectants.** MICs of antibiotics, except polymyxin B and silver sulfadiazine, were determined by the microdilution method. The MICs of polymyxin B and silver sulfadiazine were determined by the agar dilution method according to the protocols recommended by the CLSI (formerly NCCLS), standard M7-A6 (33).

Bactericidal activities of disinfectants were evaluated by time- and dose-dependent killing studies in 96-well microplates. Briefly, $10^5$ microorganisms were incubated at 35°C for 0.5 min to 60 min in 160 μl disinfectants diluted serially twofold. To neutralize the bactericidal activities of the disinfectants, a 10-μl aliquot of each suspension was transferred to 200 μl Trypticase soy broth (Becton Dickinson, Franklin Lakes, NJ) containing 15% Tween 80 (Sigma), 1% soybean lecithin (Nacalai Tesque), and 0.5% sodium thiosulfate (Nacalai Tesque) and then cultured for 24 h. The minimum bactericidal concentrations (MBCs) of disinfectants were recorded relative to the duration of incubation with bacteria.

**Transfer of drug resistance among bacteria.** Transfer of the drug resistance from *P. aeruginosa* clinical isolates to a rifampin-resistant mutant of *P. aeruginosa*, ATCC 27853 $RFP^r$, was examined with the broth mating method (25). After mating, transconjugants were selected on Mueller-Hinton agar plates containing rifampin (200 μg/ml) and imipenem (16 μg/ml) or amikacin (20 μg/ml). Plasmid DNAs from the clinical isolates were purified either with a QIAprep kit (QIAGEN, Tokyo, Japan), by Kado and Liu's (24), or method by the method of Domenico et al. (11). With the QIAprep kit or Kado and Liu's method, the bacteria were lysed at different temperatures, 22°C for 5 min or 60°C for 70 min for each method.

**PCR of class 1 integrons.** To identify the presence of a class 1 integron and to determine the size of any inserted gene cassettes, PCR amplification was performed as described previously (29) with primers 5'-cs and 3'-cs, which are specific for 5'-CS and the 3'-CS of class 1 integrons, respectively, and an Expand High Fidelity PCR system (Roche Diagnostics GmbH, Penzberg, Germany). To determine the content and order of genes in the integron, PCR amplification of the variable region of class 1 integrons was carried out with the primers listed in Table 1. All PCRs were performed with a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, Calif.). Genomic DNAs extracted as described by Sambrook and Russell (41) were used as templates. Amplification conditions were 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min or 5 min. PCR for amplicons longer than 1 kb was performed with 1.25 U of *Z-Taq* polymerase (Takara Bio) and 30 cycles of 95°C for 1 s and 68°C for 120 s according to the manufacturer's instructions.

**PCR of QRDRs.** The *gyrA*, *gyrB*, *parC*, and *parE* quinolone resistance-determining regions (QRDRs) of *P. aeruginosa* were amplified by PCR with the primers listed in Table 1 according to methods described previously (1, 21, 26, 31). PCR products were sequenced with the same primers.

**DNA sequencing.** DNA sequences were determined by the dideoxy chain termination method with an ABI PRISM 3100 sequencer (Applied Biosystems). Homology searches of nucleotide and deduced protein sequences were performed by FASTA and BLAST screens of the DDBJ, GenBank, and EMBL databases. Multiple-sequence alignments and searches for ORFs were performed with GENETYX-WIN software (Genetyx, Tokyo, Japan). The dendrogram for AACs was calculated with the CLUSTAL W Program (49).

**Cloning of the *aac(6')-Iae* gene.** The coding region of *aac(6')-Iae* (Fig. 1) was amplified by PCR with 2.5 U of *Ex Taq* DNA polymerase (Takara Bio) and primers aacS1-FC and aacS1-RC (Table 1). The PCR products were cloned into pCRT7/NT (Invitrogen) downstream of the region encoding a six-His tag. Then plasmid pAAC6, which contains *aac(6')-Iae*, or plasmid pREVAAC6, which contains *aac(6')-Iae* in the reverse direction, was transformed into *E. coli* DH5α cells by the $CaCl_2$ method (6). DNA sequences of these cloned fragments were verified by sequencing of both strands as described above.

**Purification of recombinant AAC(6')-Iae.** *E. coli* BL21-AI harboring plasmid pAAC6 was grown to an $A_{600}$ of 0.2 to 0.3 in LB medium containing 50 mg/liter ampicillin at 37°C. After addition of arabinose (final concentration, 0.02%) to induce expression of AAC(6')-Iae, the *E. coli* strain was cultured for another 18 h at 25°C. The bacterial cells were collected, resuspended in 50 mM HEPES buffer (pH 7.5) containing 0.1% Triton X-100, and lysed by sonication on ice for 15 s 40 times and then for 20 s 100 times. After centrifugation to remove the debris, the solubilized protein was applied to an AKTA Prime (Amersham Biosciences, Piscataway, NJ) system equipped with a HiTrap Chelating HP column (Amersham Biosciences) loaded with $Ni^{2+}$. The column was washed with 20 mM Tris-HCl (pH 7.9) containing 60 mM imidazole and 0.5 M NaCl and was eluted with the same buffer containing 1 M imidazole. The eluted proteins were collected and dialyzed in 50 mM HEPES buffer (pH 7.5). The protein preparation yielded a single band upon sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis (data not shown).

**Acetylation of aminoglycosides by recombinant ACC(6')-Iae.** Enzymatic acetylation of aminoglycosides was done as described previously (53). Recombinant AAC(6') from actinomycete strain #8 was provided by J. Ishikawa (National Institute of Infectious Diseases, Tokyo, Japan). Various aminoglycosides were incubated with recombinant AAC(6')-Iae or AAC(6') as a positive control in the presence of acetyl coenzyme A, and the acetylated derivatives were detected by thin-layer chromatography. The reaction was carried out at 37°C for 30 min to 12 h.

**Pulsed-field gel electrophoresis (PFGE).** Genomic DNA from *P. aeruginosa* was prepared by the procedure of Grundmann et al. (14) and digested overnight with 10 U of SpeI, XbaI, or HpaI (Takara Bio). The DNA fragments were separated on 1.0% agarose gels in 0.5× Tris-borate-EDTA buffer with a CHEF Mapper system (Bio-Rad Laboratories, Hercules, Calif.) at 6 V/cm for 20 h.

**Southern hybridization.** We performed Southern blotting to identify the location of In113. A 465-bp segment of *aac(6')-Iae* and a 362-bp segment of $bla_{IMP-1}$ amplified by PCR were labeled with horseradish peroxidase and used as probes.

**Nucleotide sequence accession number.** The nucleotide sequence of In113 reported here has been deposited in the EMBL/GenBank/DDBJ databases and assigned accession number AB104852.

## RESULTS

**Epidemiologic analysis of a nosocomial outbreak of *P. aeruginosa*.** From June 2002 to November 2002, a *P. aeruginosa* outbreak occurred in a neurosurgery ward of a 500-bed hospital in Japan. Three patients developed catheter-associated urinary tract infections with multidrug-resistant *P. aeruginosa* in June 2002. Various measures for infection control were undertaken, but four patients subsequently developed similar catheter-associated urinary tract infections with multidrug-resistant *P. aeruginosa* over the next 5 months. Seven *P. aeruginosa* isolates from these patients were analyzed by PFGE. The PFGE patterns of SpeI-, XbaI-, or HpaI-digested genomic DNAs from the isolates were identical, indicating that the isolates were all from monoclonal expansion of a single multidrug-resistant *P. aeruginosa* strain. This clone was named *P. aeruginosa* IMCJ2.S1. PFGE patterns of SpeI-, XbaI-, and

TABLE 1. PCR primers

| Primer | Sequence[a] (5'→3') | Expected size of amplicon (bp) | Position (nt)[b] | Reference or GenBank accession no. |
|---|---|---|---|---|
| 5'-cs | GGCATCCAAGCAGCAAG | | | 29 |
| 3'-cs | AAGCAGACTTGACCTGA | | | 29 |
| int1-F | TGCGTGTAAATCATCGTCGT | 838 | Downstream of *intI1* | AF071413 |
| int1-R | CGAAGTCGAGGCATTTCTGT | | 177–196 in *intI1* | AF071413 |
| IMP-F[c] | DTTYCTAAACAYGGYTTGGT | 362 | 145–164 in $bla_{IMP-1}$ | AB070224 |
| IMP-R[c] | YTTTYAGGYARCCAAACYACT | | 486–506 in $bla_{IMP-1}$ | AB070224 |
| aacS1-F | CGCAAGCTGCAGAAATTCTAT | 465 | 47–67 in *aac(6')-Iae* | This study |
| aacS1-R | TCCCATTTGCATTAGGAATCA | | 491–511 in *aac(6')-Iae* | This study |
| aadA1-F | TGATTTGCTGGTTACGGTGA | 451 | 144–163 in *aadA1* | AF071413 |
| aadA1-R | TACTGCGCTGTACCAAATGC | | 575–594 in *aadA1* | AF071413 |
| qacEdelta-F | TGAAAGGCTGGCTTTTTCTT | 286 | 2–21 in *qacEΔ1* | AF071413 |
| qacEdelta-R | GCAATTATGAGCCCCATACC | | 268–287 in *qacEΔ1* | AF071413 |
| sul-F | TCACCGAGGACTCCTTCTTC | 759 | 29–48 in *sul1* | AF071413 |
| sul-R | GGGTTTCCGAGAAGGTGATT | | 768–787 in *sul1* | AF071413 |
| int1imp1-F | AGCACCTTGCCGTAGAAGAA | 695 | 262–281 in *intI1* | AJ640197 |
| int1imp1-R | TTTTATAGCCACGCTCCACA | | 243–262 in $bla_{IMP-1}$ | AJ640197 |
| imp1aacS1-F | AAAGGCAGCATTTCCTCTCA | 737 | 265–284 in $bla_{IMP-1}$ | This study |
| imp1aacS1-R | GACGGCCAAGAATCGAAAT | | 89–107 in *aac(6')-Iae* | This study |
| aacS1aadA1-F | ATTGTGTGGTTGGGTTGGAT | 691 | 186–205 in *aac(6')-Iae* | This study |
| aacS1aadA1-R | GGAGAATCTCGCTCTCTCCA | | 231–259 in *aadA1* | This study |
| aadA1qacEd-F | TGATTTGCTGGTTACGGTGA | 873 | 144–163 in *aadA1* | AF071413 |
| aadA1qacEd-R | ATGCGGATGTTGCGATTACT | | 42–61 in *qacEΔ1* | AF071413 |
| qacEdsul-F | TCGGTGTTGCTTATGCAGTC | 306 | 167–186 in *qacEΔ1* | AF071413 |
| qacEdsul-R | ACATCCACGACGTCTGATCC | | 112–131 in *sul1* | AF071413 |
| int-R | TGCGTGTAAATCATCGTCGT | 3,172 | Downstream of *intI1* | AF071413 |
| sul-R | GGGTTTCCGAGAAGGTGATT | | 768–787 in *sul1* | AF071413 |
| sul-F | TCACCGAGGACTCCTTCTTC | 6,474 | 29–48 in *sul1* | AF071413 |
| tniB-R | ATCATCGACCTGTCCCACCT | | 16–35 in *tniBΔ1* | AF071413 |
| tniB-F | CAGAGCCAGTTGCTCCATTT | 1,749 | 395–414 in *tniBΔ1* | AF071413 |
| tniA-R | CTTTCACCGCGAAGTCACTC | | 384–403 in *tniA* | AF071413 |
| GyrA1 | TTATGCCATGAGCGAGCTGGGCAACGACT | 366 | 147–176 in *gyrA* | 26 |
| GyrA2 | AACCGTTGACCAGCAGGTTGGGAATCTT | | 484–512 in *gyrA* | 26 |
| GyrB1 | GCGCGTGAGATGACCCGCCGT | 390 | 1162–1182 in *gyrB* | 31 |
| GyrB2 | CTGGCGGTAGAAGAAGGTCAT | | 1531–1551 in *gyrB* | 31 |
| PARC1 | ATGAGCGAACTGGGGCTGGAT | 210 | 166–187 in *parC* | 21 |
| PARC2 | ATGGCGGCGAAGGACTTGGGA | | 354–375 in *parC* | 21 |
| ParE1 | CGGCGTTCGTCTCGGGCGTGGTGAAGGA | 592 | 1223–1250 in *parE* | 1 |
| ParE2 | TCGAGGGCGTAGTAGATGTCCTTGCCGA | | 1787–1814 in *parE* | 1 |
| aacS1-FC | ATGAAATACAACATTGTTAATATTA | 552 | 1–25 in *aac(6')-Iae* | This study |
| aacS1-RC | TTACATTATATTTTTCCACATTAAT | | 528–552 in *aac(6')-Iae* | This study |

[a] D stands for adenine, thymine, or guanine; R stands for adenine or guanine; Y stands for cytosine or thymine.
[b] Nucleotides are numbered according to deposited sequences.
[c] Primer designed to amplify $bla_{IMP-1}$ (accession no. AB070224) or homologous genes, including $bla_{IMP-2}$ (AJ243491), $bla_{IMP-3}$ (AB010417), $bla_{IMP-4}$ (AF445082), $bla_{IMP-5}$ (AF290912), $bla_{IMP-6}$ (AB040994), $bla_{IMP-7}$ (AF416736), $bla_{IMP-8}$ (AF322577), $bla_{IMP-9}$ (AY033653), $bla_{IMP-10}$ (AB074434), and $bla_{IMP-11}$ (AB074437).

HpaI-digested genomic DNAs from IMCJ2.S1 are shown in Fig. 2A.

**Susceptibility of *P. aeruginosa* IMCJ2.S1 to antibiotics and disinfectants.** The MICs of various antibiotics, including potent active β-lactams, against IMCJ2.S1 were compared with those against a reference strain, *P. aeruginosa* ATCC 27853 (Table 2). IMCJ2.S1 was resistant to all antibiotics tested except for arbekacin and polymyxin B. Strain ATCC 27853 was sensitive to all of the antibiotics tested except cefoxitin, flomoxef, and kanamycin. Thus, IMCJ2.S1 was classified as a multidrug-resistant strain of *P. aeruginosa*.

To test whether IMCJ2.S1 showed increased resistance to disinfectants, the MBCs of four disinfectants, povidone iodine, alkyldiaminoethylglycine hydrochloride, benzalkonium chloride, and chlorhexidine gluconate, were determined for both IMCJ2.S1 and ATCC 27853. Both strains were resistant to chlorhexidine gluconate but sensitive to povidone iodine (MBC, <0.001% [wt/vol]), alkyldiaminoethylglycine hydrochloride (MBC, <0.001% [wt/vol]), and benzalkonium choride (MBC, <0.005% [wt/vol]). The MBC patterns of these strains were identical. These results indicate that the sensitivity of IMCJ2.S1 to disinfectants is not different from that of the *P. aeruginosa* reference strain.

**Detection of an integron in *P. aeruginosa* IMCJ2.S1.** To determine if strain IMCJ2.S1 carried a class 1 integron, PCR analysis specific for class 1 integrons was performed (29). Strain IMCJ2.S1 yielded a 2.5-kbp PCR product, whereas *E. coli* CSH2 harboring plasmid NR1 (32), which carries In2 (30), yielded a 1.0-kbp PCR product. *P. aeruginosa* ATCC 27853 did not yield PCR products. These results suggest that strain IMCJ2.S1 and *E. coli* CSH2 each carry a class 1 integron and that this integron contains additional sequences that are not present in In2.

The class 1 integron frequently contains the *tniB* and *tniA* genes downstream of the 3'-CS (13, 16). To confirm the presence of a class 1 integron in IMCJ2.S1 and to elucidate the

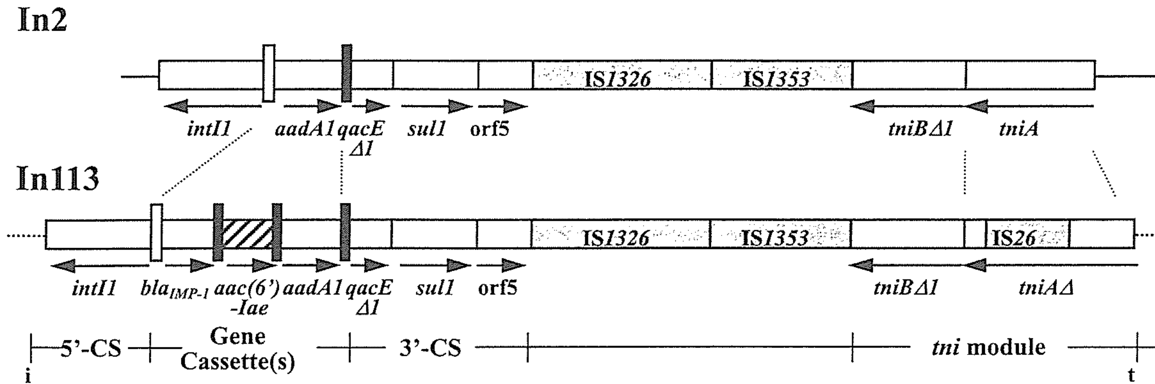


FIG. 1. Structure of In2 (GenBank accession no. AF071413) and In113. Gene cassettes are represented as open boxes with an adjacent vertical bar (59-be), shown as heavy solid vertical bars. The novel ORF found in In113 is shown as a hatched box. Genes are indicated by horizontal arrows. IS are represented as gray boxes and are labeled. The sites of the 5′-CS, gene cassettes, 3′-CS, and *tni* module are indicated just below the construct. IRi and IRt are shown as vertical lines labeled i and t, respectively, and the *attI1* sites are shown as open vertical bars toward the left of the constructs.

structure downstream of the 3′-CS, we performed PCR specific for *intI1*, *qacEΔ1*, *sulI*, and their spanning or marginal regions. PCRs yielded the expected products (Table 1), with the exception of a 4.7-kbp fragment after amplification with int-R and sul-R and a 2.5-kbp fragment after amplification with tniB-F and tniA-R. These data show that IMCJ2.S1 carries a class 1 integron and that this integron contains *intI1-sulI* in a 4.7-kbp region, *sulI-tniB* in a 6.5-kbp region, and *tniB-tniA* in a 2.5-kbp region (Fig. 1).

Identical results were obtained for the other six isolates from the outbreak.

**Structure of the class 1 integron found in *P. aeruginosa* IMCJ2.S1.** We analyzed the sequences of the PCR products to determine the structure of the class 1 integron of IMCJ2.S1. The 5′-CS contained *intI1*, the *attI1* recombination site with a 7-bp core site sequence of GTTAGAA (45), and the TGGACA (−35) and TAAACT (−10) hexamers separated by 17 bp, which is characteristic of the Pc promoter (7, 45). Although TTGTTA (−35) and TACAGT (−10) hexamers separated by 14 bp were present again downstream of the Pc promoter, this region is not likely to act as the P2 promoter, because there is no GGG sequence (7, 45).

Between the 5′-CS and 3′-CS, there were three gene cassettes (Fig. 1). The 880-nucleotide (nt) cassette contained the metallo-β-lactamase gene $bla_{IMP-1}$ (35) and a 127-nt 59-base element (59-be) site, a site for site-specific cointegration events (Fig. 3), and this cassette was identical to one described previously (2, 35). The 647-nt cassette contained an ORF and a 68-nt 59-be site (Fig. 3). The sequence of this 647-nt cassette was not found in any database, and therefore, we named this integron In113 (Fig. 1). The ORF in the 647-nt cassette encoded a 183-amino-acid (aa) product that was 55.2% identical to a 6′-*N*-aminoglycoside acetyltransferase, AAC(6′)-Ia (48), and 57.1% identical to AAC(6′)-Iq of *Klebsiella pneumoniae* (4). We named the predicted protein AAC(6′)-Iae according to the standard nomenclature (42).

AAC(6′)-Iae was relatively similar to a subfamily of AAC(6′)-I enzymes that includes AAC(6′)-Ia (48), AAC(6′)-Iq (4), and AAC(6′)-Im (19) [which is not the AAC(6′)-Im reported by Chow et al. (5) and has also been

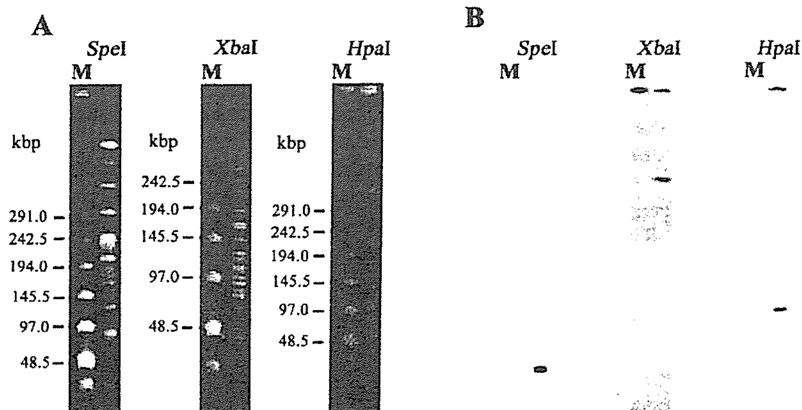


FIG. 2. (A) PFGE of SpeI-, XbaI-, and HpaI-digested genomic DNA from multidrug-resistant *P. aeruginosa* IMCJ2.S1. (B) Southern blotting of the same gels with an *aac(6′)-Iae* probe. Lanes M, HindIII-digested λ phage DNA as a size marker.

TABLE 2. In vitro susceptibilities of *P. aeruginosa* IMCJ2.S1 and *P. aeruginosa* ATCC 27853 to various antimicrobial agents

| Antibiotic | MIC (μg/ml) for: | |
|---|---|---|
| | *P. aeruginosa* IMCJ2.S1 | *P. aeruginosa* ATCC 27853 |
| Piperacillin | >128 | <4 |
| Piperacillin-tazobactam | 64 | 4 |
| Cefotaxime | >128 | 8 |
| Ceftazidime | >128 | <1 |
| Cefepime | >64 | 2 |
| Cefoxitin | >64 | >64 |
| Flomoxef | >128 | >128 |
| Moxalactam | >128 | 16 |
| Imipenem | 128 | 4 |
| Meropenem | 128 | 1 |
| Aztreonam | 128 | 2 |
| Amikacin | 128 | 2 |
| Arbekacin | 2 | <0.5 |
| Dibekacin | >128 | <0.5 |
| Gentamicin | 16 | <1 |
| Isepamicin | 128 | <4 |
| Kanamycin | >128 | >128 |
| Netilmicin | >128 | <0.5 |
| Sisomicin | >128 | <0.5 |
| Streptomycin | >64 | <4 |
| Tobramycin | 64 | <0.5 |
| Tetracycline | 32 | 16 |
| Sulfamethoxazole-trimethoprim | 128 | 32 |
| Levofloxacin | 64 | <0.5 |
| Ciprofloxacin | 32 | <0.5 |
| Polymyxin B | 2 | 2 |
| Silver sulfadiazine | 64 | 64 |

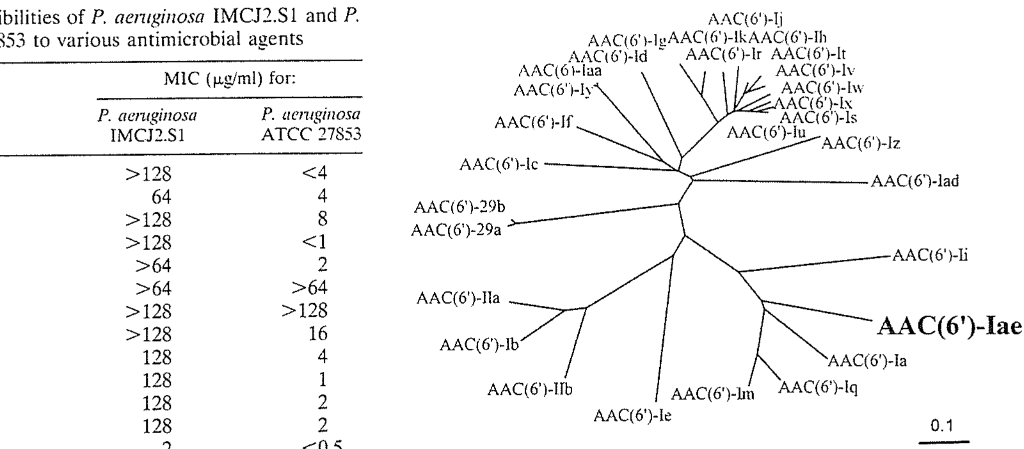


FIG. 4. Dendrogram of aminoglycoside 6′-*N*-acetyltransferases for comparison with AAC(6′)-Iae. The dendrogram was calculated with the CLUSTAL W program. Branch lengths correspond to the number of amino acid exchanges for AAC proteins. EMBL/GenBank/DDBJ accession numbers of AAC proteins are as follows: AAC(6′)-Ia, M18967-1; AAC(6′)-Ib, M23634; AAC(6′)-Ic, M94066; AAC(6′)-Id, X12618; AAC(6′)-Ie, M13771; AAC(6′)-If, X55353; AAC(6′)-Ig, L09246; AAC(6′)-Ih, L29044; AAC(6′)-Ii, L12710-1; AAC(6′)-Ij, L29045; AAC(6′)-Ik, L29510; AAC(6′)-Il, Z54241 and U13880; AAC(6′)-Im, Z54241-2; AAC(6′)-Iq, AF047556-1; AAC(6′)-Ir, AF031326; AAC(6′)-Is, AF031327; AAC(6′)-It, AF031328; AAC(6′)-Iu, AF031329; AAC(6′)-Iv, AF031330; AAC(6′)-Iw, AF031331; AAC(6′)-Ix, AF031332; AAC(6′)-Iy, AF144880; AAC(6′)-Iz, AF140221; AAC(6′)-Iaa, NC_003197; AAC(6′)-Iad, AB119105; AAC(6′)-IIa, M29695; AAC(6′)-IIb, L06163; AAC(6′)-29a, AF263519; AAC(6′)-29b, AF263519.

referred to as AAC(6′)-Ip, by Centrón and Roy (4)] (61.7% identity in a 149-aa overlap) and to AAC(6′)-Ii (9) (40.3% identity in a 166-aa overlap) (Fig. 4). On the basis of the work of Neuwald and Landsman (34), four motifs in the amino acid sequences of the subfamily proteins belonging to AAC(6′)-Iae were designated motifs C, D, A, and B (Fig. 5). Comparison of amino acid sequences of members of the AAC(6′)-I subfamily with that of AAC(6′)-Iae revealed that motifs C, D, A, and B, which are found in most GCN5-related *N*-acetyltransferases (GNATs) (12, 34), were conserved in AAC(6′)-Iae (Fig. 5). A large motif at the C terminus, motif B (12), was 63.3% identical between AAC(6′)-Im (19) and AAC(6′)-Iae. The third cassette was 856 nt long and contained the aminoglycoside 3″-adenyltransferase gene *aadA1* (18, 22) and a 60-nt 59-be site (Fig. 3). This cassette was similar to one reported previously (30, 36) except for a silent C-to-T substitution at nt 135.

The 3′-CS included *qacEΔ1* (39), *sul* (47), and orf5 (30, 37). There were three inserted sequences (IS), IS*1326* (3), IS*1353* (3), and IS*26* (38), in the region downstream of the 3′-CS (Fig. 1). IS*26* is known to be inserted into the *tniA* coding region of the *tni* transposition module (30).

**Drug resistance mediated by the AAC(6′)-Iae enzyme.** To examine the role of AAC(6′)-Iae in aminoglycoside resistance, a recombinant plasmid, pAAC6, carrying *aac(6′)-Iae* from strain IMCJ2.S1 was transformed into *E. coli* DH5α. *E. coli* harboring pAAC6 showed significantly lower susceptibility to

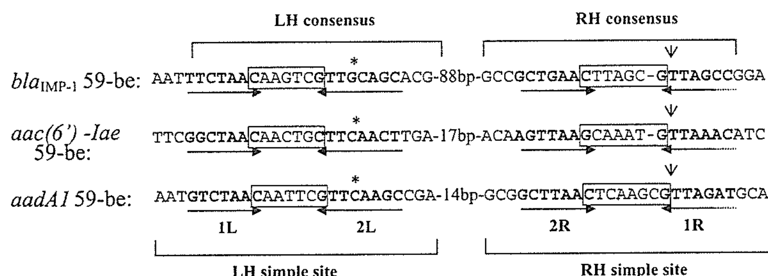


FIG. 3. Structures of 59-be of In113. Seven-base-pair putative core sites in the left-hand (LH) and right-hand (RH) consensus sequences were designated 1L and 2L and 2R and 1R, respectively. The putative recombination event occurs between the G and the first T in the 1R core site and is indicated by vertical arrows (see reference 45). The relative orientations of 1L, 2L, 2R, and 1R are indicated by arrows under the sequence. An extra base in 2L is marked with an asterisk. Inverted repeats are underscored with arrows.

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P. aeruginosa AAC(6')-Iae [AB104852]      1  MKYNIVNIKDSEKYITQAAEILFDVFSHINFDSWPSLQKAT--ETVIECISAENICIGIL 58
C. diversus AAC(6')-Ia [M18967-1]         1  MNYQIVNIAECSNYQLEAANILTEAFNDLGNNSWPDMTSAT--KEVKECIESPNLCFGLL 58
C. freundii AAC(6')-Im [Z54241-2]         1  M-------------------IKKSVLDAGNEDSWGDIKNAI--EEVEECIEHPNICLGIC 38
E. faecium AAC(6')-Ii [L12710-1]          1  M---I--I--SE-FDRNNPV-LKDQLSDLLRLTWPEEYGDSSAEEVEEMMNPERIAVAAV 51
K. pneumoniae AAC(6')-Iq [AF047556-1]     1  MDYSICDIAESNELILEAAKILRKSFLDAGNESWVDIKKAI--EEVEDCIEHPNLCLGIC 58
                                                              Motif C

P. aeruginosa AAC(6')-Iae [AB104852]     59  INDELCGWVGLREMYKKT-WELHPMVIKKKHQNKGFGKILIFETEKKAKERNLEGIVLGT 117
C. diversus AAC(6')-Ia [M18967-1]        59  INNSIVGWIGLRPMYKET-WELHPLVVRPDYQNKGIGKILLKELENRAREQGIIGIALGT 117
C. freundii AAC(6')-Im [Z54241-2]        39  LDDKLIGWTGLRPMYDKT-WELHPMVIKTEYQGKGFGKVLLRELETRAKSRGIIGIALGT 97
E. faecium AAC(6')-Ii [L12710-1]         52  DQDELVGFIGAIPQYGITGWELHPLVVESSRRKNQIGTRLVNYLEKEVASRGGITIYLGT 111
K. pneumoniae AAC(6')-Iq [AF047556-1]    59  LDDKLIGWTGLRPMYDKT-WELHPMVIKTEYQCRGIGKVLIKELEKRAKGRGIIGIALGT 117
                                             Motif D                   Motif A

P. aeruginosa AAC(6')-Iae [AB104852]    118  DDETFRITLSMSELNNENIFHEIKNIKNLKNHPFEFYEKCGYSIIGVIPNANGKNKPDIL 177
C. diversus AAC(6')-Ia [M18967-1]       118  DDEYYRISLSLITITEDNIFDSIKNIKNINKHPYEFYQKNGYYIVGIIPNANGKNKPDIW 177
C. freundii AAC(6')-Im [Z54241-2]        98  DDEYQKISLSMIDINERNIFDEIGNIKNVNNHPYEFYKKCGYMIVGIIPNANGKRKPDIW 157
E. faecium AAC(6')-Ii [L12710-1]        112  DDLDHGITLSQTDLYVHT-FDKVASIQNLREHPYEFYEKLGYKIVGVLPNANGWDKPDI- 169
K. pneumoniae AAC(6')-Iq [AF047556-1]   118  DDEYQKISLSMIDINERNIFDEIGNIKNVTNHPYEFYKKCGYMIVGIIPNANGKRKPDIW 177
                                                              Motif B

P. aeruginosa AAC(6')-Iae [AB104852]    178  MWKNIM                                                       183
C. diversus AAC(6')-Ia [M18967-1]       178  MWKSLIKE                                                     185
C. freundii AAC(6')-Im [Z54241-2]       158  MWQILARKNSP-TIAST                                            173
E. faecium AAC(6')-Ii [L12710-1]        170  -WMAKTIIPRPDSQ                                               182
K. pneumoniae AAC(6')-Iq [AF047556-1]   178  MWKDIS                                                       183
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FIG. 5. Alignment of the AAC(6')-Iae amino acid sequence with those of four members of the AAC(6')-I subfamily. Identical residues are marked with black boxes. Four motifs, including the highly conserved motif B, are underlined. A conserved region of 21 amino acids, described by Shmara et al. (44), is indicated by a dotted line. GenBank accession numbers are given in brackets to the right of AAC names. *C. diversus*, *Citrobacter diversus*; *C. freundii*, *Citrobacter freundii*.

amikacin, dibekacin, isepamicin, kanamycin, netilmicin, sisomicin, and tobramycin than the parent strain and the negative control. MICs for other aminoglycosides, including arbekacin, gentamicin, and streptomycin, were unchanged (Table 3). These results indicate that *aac(6')-Iae* is involved in aminoglycoside resistance.

To examine potential acetylase activity of AAC(6')-Iae, we assessed the purified recombinant AAC(6')-Iae against aminoglycosides by thin-layer chromatography (53). As shown in Fig. 6, kanamycin, amikacin, tobramycin, netilmicin, sisomicin, isepamicin, arbekacin, neomycin, and gentamicin were acetylated by AAC(6')-Iae and AAC(6'). Acetylation by AAC(6')-Iae was complete for all of these aminoglycosides except gentamicin, which showed incomplete acetylation. These aminoglycosides all have 6'-$NH_2$. The present results, therefore, suggest that AAC(6')-Iae is a functional acetyltransferase that modifies the 6'-$NH_2$ position of aminoglycosides.

**Location of In113.** Clinical isolates of *P. aeruginosa* frequently possess the R plasmid, which carries a class 1 integron. Therefore, we screened our seven *P. aeruginosa* clinical isolates for the presence of this plasmid. *P. aeruginosa* GN17203 was used as a positive control for $bla_{IMP-1}$, since it has been shown to harbor pMS350, which contains a $bla_{IMP-1}$ gene. Genomic DNA from IMCJ2.S1 was used as a control for *aac(6')-Iae* and $bla_{IMP-1}$.

The extracts from the seven clinical isolates and *P. aeruginosa* GN17203 were separated by agarose gel electrophoresis, and Southern blotting with *aac(6')-Iae* or $bla_{IMP-1}$ as a probe was performed. A plasmid that contained $bla_{IMP-1}$ but not *aac(6')-Iae* was detected in *P. aeruginosa* GN17203. Despite

TABLE 3. Aminoglycoside resistance patterns of *E. coli* DH5α alone or harboring plasmids with or without *aac(6')-Iae*

| Strain | MIC (μg/ml)[a] of: | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | AMK | ABK | DIB | GEN | ISE | KAN | NEO | NET | SIS | STR | TOB |
| *E. coli* DH5α(pAAC6)[b] | 8 | 0.5 | 32 | 0.25 | 8 | 64 | 4 | 32 | 16 | 4 | 8 |
| *E. coli* DH5α(pREVAAC6)[c] | 0.5 | 0.5 | 0.5 | 0.25 | 0.25 | 1 | 2 | 0.25 | 0.25 | 4 | 0.5 |
| *E. coli* DH5α(pCRT7/NT)[d] | 0.5 | 0.25 | 0.5 | 0.25 | 0.25 | 1 | 2 | 0.25 | 0.25 | 4 | 0.25 |
| *E. coli* DH5α | 0.5 | 0.5 | 0.5 | 0.25 | 0.25 | 1 | 2 | 0.25 | 0.25 | 4 | 0.25 |

[a] AMK, amikacin; ABK, arbekacin; DIB, dibekacin; GEN, gentamicin; ISE, isepamicin; KAN, kanamycin; NEO, neomycin; NET, netilmicin; SIS, sisomicin; STR, streptomycin; TOB, tobramycin.
[b] Recombinant plasmid constructed by cloning *aac(6')-Iae* into pCRT7/NT.
[c] Recombinant plasmid constructed by insertion of DNA fragment with reverse sequence of *aac(6')-Iae* into pCRT7/NT.
[d] Cloning vector (ABPC).

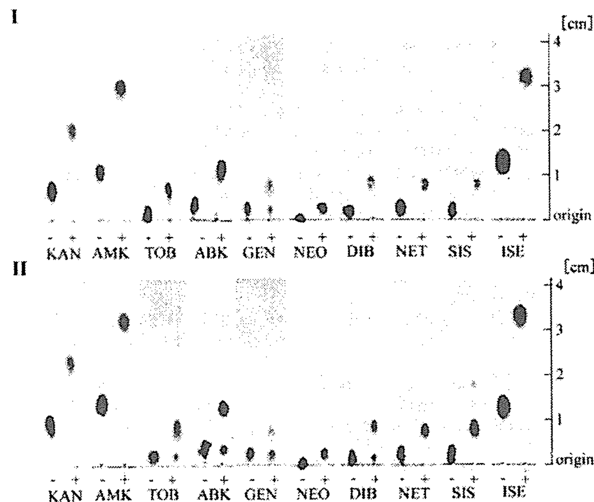


FIG. 6. Thin-layer chromatogram of aminoglycosides incubated with AAC(6′)-Iae protein (I) or with AAC(6′) from *Streptomyces lividans* TK21 as a control (II) (53) in the presence (+) or absence (−) of acetyl coenzyme A. KAN, kanamycin; AMK, amikacin; TOB, tobramycin; ABK, arbekacin; GEN, gentamicin; NEO, neomycin; DIB, dibekacin; NET, netilmicin; SIS, sisomicin; ISE, isepamicin.

repeated attempts (three times per procedure), we did not detect this plasmid by ethidium bromide staining or Southern blotting in any of the clinical isolates (data not shown). In contrast, Southern hybridization of SpeI-, XbaI-, and HpaI-digested genomic DNAs of the seven clinical isolates revealed 50-kb, 250-kb, and 60-kb *aac(6′)-Iae*-positive fragments, respectively (Fig. 2). These fragments were also positive for $bla_{IMP-1}$ (data not shown). To examine whether the drug-resistant phenotype of *P. aeruginosa* IMCJ2.S1 can be transferred by conjugation, IMCJ2.S1 was incubated with *P. aeruginosa* ATCC 27853 RFP$^r$. Carbapenem resistance was transferred from *P. aeruginosa* GN17203 to *P. aeruginosa* ATCC 27853 RFP$^r$, consistent with the results reported by Watanabe et al. (51). In contrast, resistance to amikacin or carbapenem was not transferred from IMCJ2.S1 to ATCC 27853 RFP$^r$. These results suggest that In113 is located in the chromosome, and not on a plasmid, of *P. aeruginosa* IMCJ2.S1.

**Resistance of IMCJ2.S1 to fluoroquinolones.** IMCJ2.S1 was highly resistant to fluoroquinolones (Table 2). This resistance is typically associated with mutations in the QRDR within *gyrA*, *gyrB*, *parC*, and *parE*, which encode DNA gyrase or topoisomerase IV in *P. aeruginosa* (1, 21, 26, 31). Therefore, we screened IMCJ2.S1 mutations within the QRDR. Compared to the *gyrA* sequence of strain PAO1 (46), the *gyrA* sequence of IMCJ2.S1 contained an ACC-to-ATC mutation in codon 83 that causes a Thr-to-Ile change in the A subunit of DNA gyrase. IMCJ2.S1 also had a TCG-to-TTG mutation in codon 87 of *parC* that causes a Ser-to-Leu substitution in the C subunit of topoisomerase IV. IMCJ2.S1 had four mutations in *gyrB*: CGC to CGT in codon 396, AAA to AAG in codon 408, GAA to GAG in codon 484, and TTG to CTG in codon 513. There were four mutations in *parE*: GAA to GAG in codon 448, GGT to GGC in codon 472, AGT to AGC in codon 474, and GCC to GCT in codon 477. These mutations in *gyrB* and *parE* did not lead to amino acid changes in the proteins encoded (1, 31). Identical results were obtained with the other six clinical isolates. Together, these results indicate that IMCJ2.S1 contains mutations in *gyrA* and *parC* that are associated with its fluoroquinolone resistance.

## DISCUSSION

A variety of aminoglycoside 6′-*N*-acetyltransferases have been described (Fig. 4) and classified into three subgroups (42, 50). Recently, a new enzyme, AAC(6′)-Iad, which is a member of the largest subfamily, was isolated from an *Acinetobacter* genospecies 3 strain in Japan (10). In the present study, we identified AAC(6′)-Iae, which shows considerable phylogenetic distance from members of the largest subfamily, which includes AAC(6′)-Iad and its divergents (Fig. 4). AAC(6′)-Iae belongs to the subfamily comprising AAC(6′)-Ia, -Ii, -Im, and -Iq (4, 9, 19, 48). There was only a low level of homology between the 59-be site of *aac(6′)-Iae* and those of the genes encoding other members of the *aac(6′)-I* family. Furthermore, *aac(6′)-Iae* has a low G+C content (26.8%) (data not shown), whereas the average G+C content of the *P. aeruginosa* PAO1 genome is 66.6% (46). Therefore, *aac(6′)-Iae* may be derived from an environmental species with an intrinsically low G+C content.

AAC(6′)-Iae from *P. aeruginosa* strain IMCJ2.S1, which was responsible for an outbreak of catheter-associated urinary tract infections, acetylated all of the aminoglycosides with 6′-$NH_2$, and acetylation of arbekacin and neomycin appeared to be complete (Fig. 6I). However, *E. coli* DH5α(pAAC6), expressing exogenous AAC(6′)-Iae, was sensitive to arbekacin and did not show reduced susceptibility to neomycin. Arbekacin and neomycin were shown to retain their antibiotic effects even after they were acetylated by AAC(6′) from an arbekacin-resistant actinomycete strain at the 6′ positions (53). *Enterococcus faecium* producing AAC(6′)-Ii was susceptible to neomycin even though AAC(6′)-Ii acetylated neomycin (52). These results suggest that acetylation of arbekacin and neomycin at 6′ positions does not affect the antimicrobial activities of these drugs. We cannot exclude the possibility that the antimicrobial activity observed after treatment with AAC(6′)-Iae is due to residual arbekacin or neomycin that was not acetylated.

*E. coli* DH5α expressing AAC(6′)-Iae was sensitive to gentamicin (Table 3), although AAC(6′)-Iae showed only partial acetylation of gentamicin (Fig. 6I). The sensitivity of these bacteria to gentamicin appears to be due to incomplete acetylation of gentamicin, which was observed with AAC(6′) from an arbekacin-resistant actinomycete strain (53)(Fig.6II). Commercially available gentamicin is a mixture of a number of derivatives of gentamicin, such as gentamicin $C_1$, $C_{1a}$, $C_2$, and $C_{2b}$, that have modifications of position 6′. Gentamicin $C_1$ and $C_{2b}$ carry a methyl group on N-6′ and are refractory to AAC(6′)-I enzymes (42, 50). We cannot exclude the possibility that acetylated gentamicin components, which are more susceptible to AAC(6′)-I enzymes, retain antibiotic activity.

In the present study, we identified In113, a class 1 integron that contains a novel aminoglycoside resistance gene, *aac(6′)-Iae*. Several classes of integrons have been categorized on the basis of the structure of integrase (15, 40). The most common integrons in *P. aeruginosa* are those of class 1 (27, 28, 37).

Because their structures are very similar to each other, the direct origin of In113 could be from In2 (30), which was originally isolated from *Shigella flexneri* in Japan in the late 1950s (32) (Fig. 1).

IMCJ2.S1 was resistant to all antibiotics tested except arbekacin and polymyxin B (Table 2). However, the presence of In113 and the mutations in *gyrA* and *parC* of the QRDR are not sufficient to explain the multidrug resistance of this strain. Alterations of *gyrA* and *parC* are known to contribute to fluoroquinolone resistance (1, 21, 26, 31). The $bla_{IMP-1}$ gene cassette, which encodes the IMP-1 metallo-β-lactamase, confers resistance to all β-lactams except monobactams (2, 27, 35). The *aac(6')-Iae* gene cassette, which encodes AAC(6')-Iae, confers resistance to amikacin, dibekacin, isepamicin, kanamycin, netilmicin, sisomicin, and tobramycin (Table 3). The variant *aadA1* gene cassette, which encodes aminoglycoside 3″-adenylyltransferase, confers resistance to streptomycin (18, 22). The *sulI* gene, which encodes dihydropteroate synthetase type I, confers resistance to sulfamethoxazole (47). Thus, the resistance of IMCJ2.S1 to aztreonam, gentamicin, tetracycline, trimethoprim, and silver sulfadiazine appears to be related to another, unidentified resistance factor(s).

In conclusion, we describe here a novel aminoglycoside 6'-*N*-acetyltransferase gene contained on a class 1 integron in a *P. aeruginosa* strain that caused a nosocomial outbreak of urinary tract infections. In113 may spread across Japan, because β-lactams, including carbapenems and aminoglycosides, are frequently used as therapeutic agents against *P. aeruginosa* and methicillin-resistant *Staphylococcus aureus* (20, 23). Surveillance for multidrug-resistant *P. aeruginosa* containing In113 is under way at several medical care facilities in the Sendai area of Japan.

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# Cloning and Characterization of a Novel Trimethoprim-Resistant Dihydrofolate Reductase from a Nosocomial Isolate of *Staphylococcus aureus* CM.S2 (IMCJ1454)

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**A novel gene, *dfrG*, encoding a trimethoprim (TMP)-resistant dihydrofolate reductase (DHFR, designated S3DHFR) was cloned from a clinical isolate of methicillin-resistant *Staphylococcus aureus*. *Escherichia coli* expressing *dfrG* was highly resistant to TMP. Recombinant S3DHFR exhibited DHFR activity that was not inhibited by TMP.**

Trimethoprim (TMP) is a potent inhibitor of bacterial dihydrofolate reductase (DHFR) and is effective in vitro against methicillin-resistant *Staphylococcus aureus* (MRSA). In combination with sulfamethoxazole, TMP has been used successfully to treat patients infected with MRSA and is effective at eradicating carriage (10, 16). Resistance of *S. aureus* to TMP was first reported in the 1980s (12) and was found to be due to plasmid-mediated production of an additional DHFR that was less sensitive to TMP than intrinsic DFHR (*S. aureus* DHFR [SaDHFR]) encoded by the *dfrB* gene on the chromosome (1, 12). Plasmid-mediated production of an additional TMP-resistant DHFR is one of the most common mechanisms of resistance to TMP in bacterial organisms. At least 14 different types of TMP-resistant DHFRs in gram-negative bacteria have been reported (10); however, only a limited number of TMP-resistant DHFRs in gram-positive bacteria have been reported (10).

A total of 43 clinical isolates of MRSA from Chiang Mai, Thailand, and 244 clinical isolates of MRSA from Tokyo, Japan, were analyzed in this study. All isolates were positive for *dfrB* by PCR and also positive for *femB* encoding coagulase and for *mecA* associated with methicillin resistance. All isolates from Chiang Mai, Thailand, were resistant to TMP, whereas all those from Tokyo, Japan, except one, *S. aureus* IMCJ934, were sensitive to TMP (Table 1). Crude extracts prepared from a TMP-resistant isolate from Chiang Mai, *S. aureus* CM.S2 (IMCJ1454), showed DHFR activity, and $K_m$ values of the extract for DHF and NADPH were similar to those of crude extracts from TMP-sensitive strain ATCC 25923 (Table 2); however, the 50% inhibitory concentration ($IC_{50}$) of TMP for the crude extract of strain CM.S2 was more than 15,000-fold greater than that of ATCC 25923.

HindIII-digested fragments of the *S. aureus* CM.S2 genome were cloned, transformed into *Escherichia coli* DH5α cells, and selected on agar medium containing TMP (8 μg/ml). The resultant plasmid, named pSA1, had a 3.5-kb insert containing a complete open reading frame (ORF) surrounded by truncated ORFs (data not shown). The complete ORF consisted of 498 bp encoding a putative protein of 165 amino acids with similarities to TMP-resistant DHFR from *Staphylococcus haemolyticus* (79% identity) (7), *Bacillus anthracis* (67% identity) (2), and *Bacillus cereus* (65% identity) (15) (Fig. 1). The deduced

TABLE 1. MICs of trimethoprim in *S. aureus* and *E. coli* strains

| Strain | MIC of TMP (μg/ml) | Characteristic(s) or genotype |
|---|---|---|
| *S. aureus* CM.S2 (IMCJ1454) | >512 | Clinical isolate from Chiang Mai, Thailand, in 2003 |
| *S. aureus* IMCJ934 | >512 | Clinical isolate from Tokyo, Japan, in 2001 |
| *S. aureus* ATCC 29213 | 4 | Quality control strain for antimicrobial susceptibility testing |
| *E. coli* DH5α(pSA1) | >512 | Transformant harboring a 3.5-kb BamHI fragment with *dfrG* ligated to pHSG398 |
| *E. coli* DH5α(pHSG398) | <2 | Transformant harboring pHSG398 |
| *E. coli* DH5α(pT7dfrG) | >512 | Transformant harboring PCR-amplified *dfrG* ligated to pCRT7/NT |
| *E. coli* DH5α(pT7dfrB) | 128 | Transformant harboring PCR-amplified intrinsic *dfrB* ligated to pCRT7/NT |
| *E. coli* DH5α(pCRT7/NT) | <2 | Transformant harboring pCRT7/NT |
| *E. coli* DH5α | <2 | *supE44 hsdR17 recA1 gyrA96 endA1 thi-1 relA1* |

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TABLE 2. Enzyme kinetic and inhibitory properties of staphylococcal DHFRs

| DHFR | Origin | $K_m$ (μM) | | $IC_{50}$ of TMP (μM) |
|---|---|---|---|---|
| | | DHF | NADPH | |
| Crude enzyme | *S. aureus* CM.S2 (IMCJ1454) | 5.83 ± 2.09 | 15.17 ± 1.73 | 214 |
| | *S. aureus* ATCC 25923 | 3.16 ± 1.99 | 14.78 ± 2.73 | 0.013 |
| TMP-resistant DHFRs | | | | |
| S3DHFR | *S. aureus* CM.S2 (IMCJ1454) | 2.68 ± 1.09 | 2.38 ± 1.97 | 254 |
| S2DHFR[a] | *S. haemolyticus* MUR313 | 5.1 | 1.7 | 127 |
| S1DHFR[a] | *S. aureus* | 6.6 | 12.4 | 9.8 |
| TMP-sensitive DHFRs | | | | |
| $SaDHFR_{CM.S2}$ | *S. aureus* CM.S2 (IMCJ1454) | 3.01 ± 1.40 | 2.97 ± 0.57 | 0.014 |
| SaDHFR[a] | *S. aureus* ATCC 25923 | | | 0.012 |

[a] Data from references 6, 7, and 8.

protein is somewhat less similar to the intrinsic TMP-sensitive DHFRs from *S. aureus* (SaDHFR) (8), *S. epidermidis* (SeDHFR) (6), and *E. coli* K-12 (17), with 41%, 40%, and 40% similarity, respectively (Fig. 1). This complete ORF was named *dfrG*, and the deduced protein was designated S3DHFR. Amino acid sequence alignment of DHFRs suggests that residues involved in the binding of TMP and NADPH in other DHFRs are conserved in S3DHFR (Fig. 1). An ORF downstream of *dfrG*, designated *orfU1*, was located in the opposite direction of *dfrG* and consists of 1,950 bp encoding 650 amino acids, although the deduced amino acid sequence did not show any significant homology to sequences of other previously reported proteins. An ORF upstream of *dfrG* consisted of 582 nucleotides and was identical to the 3′-flanking region of the *SAV0404* gene encoding a hypothetical protein (11). *dfrG* and *orfU1* were flanked by a 28-bp inverted repeat and a 7-bp direct repeat,

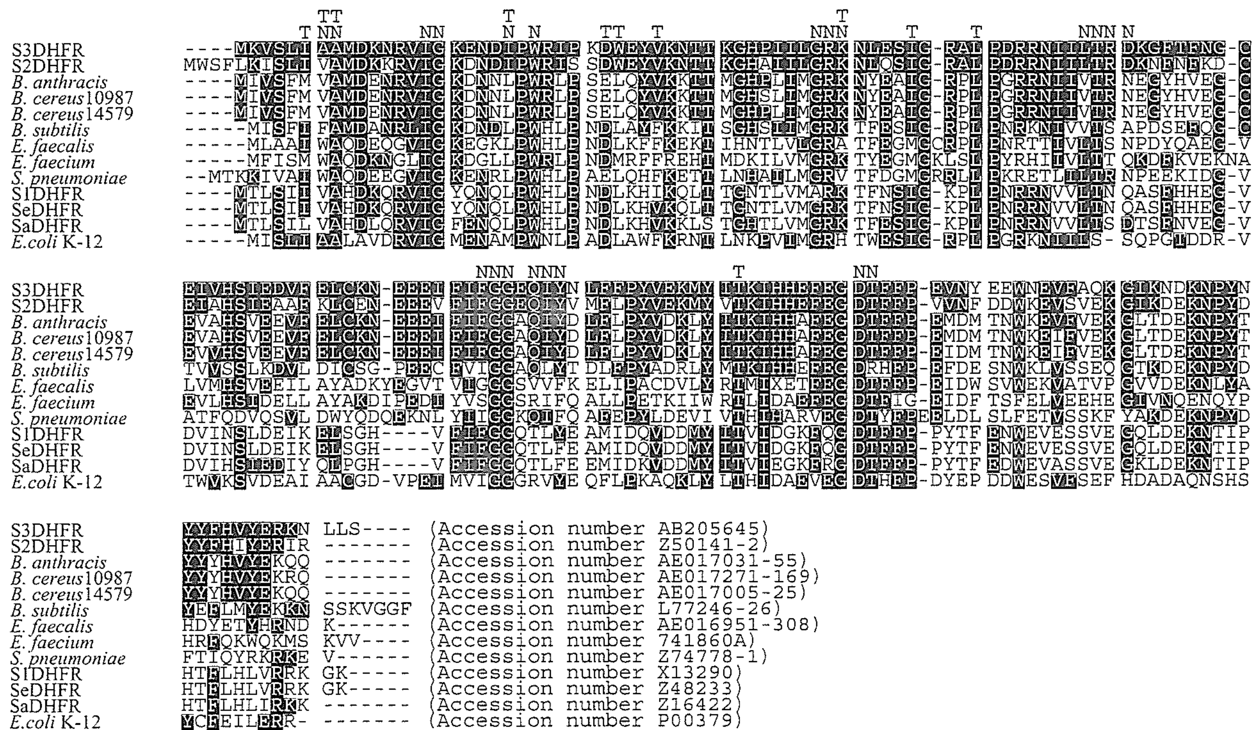


FIG. 1. Multiple-sequence alignment of the amino acid sequence of S3DHFR from *S. aureus* CM.S2 (IMCJ1454) isolate with those of DHFRs from other bacteria. The amino acid sequence of S3DHFR was compared with that of type S1 from *S. aureus*, S2 from *S. haemolyticus* MUR313, and the chromosomal DHFRs from *B. anthracis* Ames, *B. cereus* ATCC 10987, *B. cereus* ATCC 14579, *Bacillus subtilis* Marburg, *E. faecalis* V583, a methotrexate-resistant mutant of *E. faecium* strain A, *Streptococcus pneumoniae* ATCC 49619, *Staphylococcus epidermidis* ATCC 14900 (SeDHFR), *S. aureus* ATCC 25923 (SaDHFR), and *E. coli* K-12. Sequence comparison was performed by aligning the proteins with the ClustalW program (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html). Amino acid positions involved in the binding of trimethoprim (T) and NADPH cofactor (N) are according to studies of the *E. coli* K-12 enzyme (3, 9, 13, 14). Identical residues are indicated by white letters on black background. Gaps introduced to maximize alignment are indicated by dashes.