(Catalog No. PK-6102; Vector Laboratories; Burlingame, CA), slides were incubated for 20 min at room temperature in blocking solution (5% horse serum-PBS for uPAR, PAI-1, CAP18, IL-8 CD14, and cytokeratin; 5% goat serum for CD15) and washed thrice with PBS. Slides were then incubated in avidin blocking solution (Catalog No. SP-2001; Avidin/Biotin Blocking Kit; Vector Laboratories) for 15 min at room temperature, washed thrice with PBS for surface staining, and then washed in PBS-0.05% Tween 20–0.01% Thimerosal for cytoplasmic staining. Slides were then incubated with biotin blocking solution for 15 min at room temperature and washed thrice with PBS-0.05% Tween 20–0.01% Thimerosal. Following this preparation, slides were incubated with primary monoclonal antibody (for uPAR, Catalog No. 3936; American Diagnostica; Greenwich, CT; for PAI-1, Catalog No. 3785; American Diagnostica; for CAP18, see primary antibody, ELISA methods above; for IL-8, Catalog No. 554717; BD Pharmingen mouse IgG2b clone G265–8; for CD14, Catalog No. 555396; BD Pharmingen; and for cytokeratin, Catalog No. 349205; BD Pharmingen) each diluted in 5% horse serum-1% BSA-0.05% Tween 20-PBS. For CD15, the antibody (Catalog No. 555400; BD Pharmingen) was diluted in 5% normal goat serum-1%BSA-0.05% Tween 20-PBS. Slides were incubated with primary antibody for 60 min at room temperature, and washed five times with PBS-0.05% Tween 20–0.01% Thimerosal. An isotype control antibody was run with each experiment (mouse IgG isotype control; Catalog No. I-2000; Vector Laboratories). For staining uPAR, PAI-1, CAP18, IL-8, CD14, and cytokeratin, reactions were developed with biotinylated horse anti-mouse antibody (Catalog No. PK-6102; Vector Laboratories) diluted in 5% horse serum-1% BSA-0.05% Tween 20-PBS. For staining CD15, the reaction was developed with biotinylated goat anti-mouse antibody (Catalog No. PK-6102; Vector Laboratories) diluted in 5% goat serum-1% BSA-0.05% Tween 20-PBS. Slides were incubated for 30 min at room temperature and washed five times with PBS-0.05% Tween 20–0.01% Thimerosal. For development, slides were incubated for 30 min at room temperature with Vector ABC reagent (Catalog No. PK-6102; Vector Laboratories) prepared in PBS 30 min before use and then washed thrice in PBS. Color was developed (3,3'-diaminobenzidine peroxidase substrate; Catalog No. SK-4100; or Vector VIP [peroxidase substrate, Catalog No. SK-4600; Vector Laboratories]) according to manufacturer's instruction. Color development was stopped by washing in distilled water, the slides were air dried, and the slides were viewed under microscope to assess the positive staining cells and determine cell type by comparing with Wright-Giemsa–stained slides (Hema 3 set, Catalog No. 122–911; Biochemical Science; Swedesboro, NJ).

Flow Cytometry

Five × 10^5 Sputolysin-treated (Calbiochem Corporation; San Diego, CA), PBS-washed sputum cells were added to each assay tube, incubated in 5% BSA-PBS for 10 min at room temperature to block nonspecific binding, washed once in PBS, fixed in 4% formaldehyde-PBS for 15 min at room temperature, and washed once with PBS. The cell samples were permeabilized in 0.5% saponin-PBS for 10 min at room temperature and washed once in 0.1% saponon-0.5% BSA-PBS. Monoclonal anti-human IL-8, uPAR, PAI-1, and CAP18 antibodies, and mouse IgG1, G2a, and G2b isotype control antibodies were labeled with fluorescein isothiocyanate (FITC) according to the zenon complex formation protocol (Zenon Alexa Fluor 488 mouse IgG1, IgG2a and IgG2b labeling kits, Catalog Nos. Z-25002, Z-25202, and Z-250102; Molecular Probes; Eugene, OR). Phycoerythrin (PE) labeled anti-human CD14, CD15, and cytokeratin were obtained from BD Pharmingen (Catalog Nos. 555398, 555402, and 347204, respectively). FITC- and PE-labeled anti-human antibodies (IL-8/FITC, uPAR/FITC, PAI-1/FITC, CAP18/FITC, CD15/PE, CD14/PE, and CK/PE) were added to respective tube with 1% mouse serum (Sigma Chemical), to reduce nonspecific staining. Mouse IgG1, IgG2a, IgG2b (Southern Biotech; Birmingham, AL), and IgM (Catalog No. 555584; BD Pharmingen) isotype-matched controls were included in each experiment. Tubes were incubated for 30 min at room temperature in the dark with slowing mixing followed by one wash with PBS and resuspension of the pellet in 300 μL of 1% formaldehyde. Samples were analyzed using a flow cytometer (FACSCalibur E3139; Becton Dickinson; Franklin Lakes, NJ).

Statistical Analysis

Data are expressed as mean ± SE unless otherwise indicated. Statistical comparisons were made using Student t test for unpaired two-group samples, analysis of variance for multiple group comparisons, and Pearson correlation coefficient. Two-tailed tests were performed, and a p value of < 0.05 was considered significant.

Results

Subject Groups

The CF patients (17 women and 11 men) were generally young adults (mean ± SD age, 23.7 ± 11.1 years) with well-preserved pulmonary function (mean ± SD FEV_1, 74.0 ± 17.4% predicted). The patients with COPD (24 women and 50 men) were, as expected, older (59.2 ± 9.9 years) with generally greater airflow obstruction (FEV_1, 54.6 ± 13.7% predicted). The patients with asthma (19 women and 15 men) were intermediate between these other groups in age (47.4 ± 13.9 years) and airflow obstruction (FEV_1, 65.9 ± 13.0% predicted). All but two patients with asthma were receiving regular bronchodilator medication (β_2-adrenergic aerosols and/or theophylline), while only seven patients were receiving regular inhaled corticosteroids.

CAP18 in Serum, BAL Fluid, and Sputum

To study the biology of CAP18 in CF, in a pilot study we first measured serum CAP18 levels by ELISA in patients with CF in stable condition (n = 15) and compared these to samples obtained from CF patients on admission to hospital for treatment of pulmonary exacerbation (n = 15) and 15 healthy adult control subjects. The ELISA showed linear parallelism in the range of 3 to 3,000 ng/mL with interassay coefficient of variation < 15%. Levels of CAP18 were similar in the three subject groups (stable CF, 966 ± 980 ng/mL; exacerbation CF, 1,137 ± 685 ng/mL; and control, 1,012 ± 648 ng/mL). We next compared CAP18 levels in BAL fluid of 23 patients with CF and 12 control subjects. CF patients had significantly higher levels of BAL fluid CAP18 than control subjects (189.7 ± 18.7 ng/mL vs

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120.7 ± 24.7 ng/mL, $p = 0.036$, by two-sided unpaired t test). As this suggested increased local production and/or reduced metabolism within the pulmonary compartment of inflamed CF airways, we proceeded to measure CAP18 levels in expectorated sputum of 30 patients with CF. Expectorated CF sputum levels (177.4 ± 14.7 ng/mL) were quite similar those in BAL, suggesting that sputum is an easily accessible and representative sample of airway secretions for measurement of CAP18 levels.

In order to obtain sputum samples from CF patients with mild disease severity and little or no sputum productivity (including nonexpectorators), we then proceeded to study CAP18 levels in induced-sputum samples using a validated standardized methodology. All nonexpectorators were able to produce adequate sputum for analysis after induction. CF patients were compared to patients with COPD, patients with asthma, and healthy control subjects. CF and COPD patients had comparable levels (79.6 ± 93 ng/mL vs 75.3 ± 38.9 ng/mL, respectively) and significantly elevated levels of sputum CAP18 when compared to control subjects (39.9 ± 24.2 ng/mL, $p < 0.009$ for either group vs control), while asthmatics had significantly reduced CAP18 levels (13.6 ± 9.8 ng/mL) compared to control subjects ($p < 0.0001$) or patients with CF or COPD ($p < 0.0001$) [Table 1].

Cytokines and Plasmin Activation Component Levels in Induced Sputum of CF, COPD, and Asthma Patients, and Control Subjects

To see if this pattern was similar to other innate defense factors, we compared levels of IL-8, IL-10, INF-γ, TNF-α, uPA, uPAR, and PAI-1 in these subjects. As also shown in Table 1, levels of sputum IL-8 were significantly higher in patients with CF than in patients with COPD or asthma ($p < 0.001$ for each) or control subjects ($p < 0.0001$); COPD and asthma patients also had higher IL-8 levels than control subjects ($p < 0.05$). IL-10 levels were also higher in the CF patients than patients with COPD or asthma, or control subjects ($p < 0.05$), without differences noted between the latter three groups. TNF-α levels showed a similar pattern as IL-10 with elevation in patients with CF compared to patients with COPD or asthma, or control subjects ($p < 0.001$). INF-γ levels were similar among all four groups.

uPA levels were similar among the four groups. In contrast, uPAR levels were elevated and similar in patients with CF and COPD compared to patients with asthma and control subjects ($p < 0.001$ for each); uPAR levels were also higher in asthma than controls ($p < 0.05$). PAI-1 levels were comparably elevated in patients with CF, COPD, and asthma, compared to control subjects ($p < 0.05$; Table 1).

Neutrophil Origin of Sputum CAP18, IL-8, and PAI-1

In order to examine the cellular sources of these sputum substances in patients with CF, we examined cells obtained from induced-sputum samples by immunohistochemical staining and independently by flow cytometry. Most sputum cells were either intact or more commonly degenerated polymorphonuclear neutrophils with variable mucus component and occasional other cell types such as monocytes, macrophages, eosinophils, or squamous epithelial cells; ciliated epithelial cells were not seen (Fig 1, *top*). CAP18 heavily stained the cytoplasmic granules of these neutrophils (Fig 1, *center* and *bottom*), suggesting that a major source of sputum CAP18 was infiltrating neutrophils. CAP18 is known to be localized to secondary or specific neutrophil granules. Using flow cytometry, CAP18 expression was markedly increased in airway cells that coexpressed CD15 (3-fucosyl-N-acetyl-lactosamine), which is present in the intracellular secondary granules of neutrophils,

Table 1—*Levels of Innate Immune Factors in Sputum of Patients With CF, COPD, and Asthma, and Healthy Control Subjects**

Factors, pg/mL	CF (n = 28)	COPD (n = 74)	Asthma (n = 34)	Healthy (n = 44)
CAP18	79,623 ± 18,597†	75,262 ± 4,583†	13,646 ± 1,678†	39,936 ± 3,644
IL-8	29,880 ± 4,226†	11,852 ± 1,880†	11,683 ± 3,707†	1,713 ± 317
IL-10	386 ± 87†	53 ± 14	36 ± 4	72 ± 14
INF-γ	794 ± 137	392 ± 65	627 ± 225	684 ± 117
TNF-α	74 ± 31†	10 ± 4	8 ± 2	12 ± 6
uPA	59 ± 26	76 ± 24	64 ± 30	18 ± 6
uPAR	803 ± 112†	583 ± 87†	399 ± 103†	85 ± 11
PAI-1	3,812 ± 469†	6,410 ± 1039†	4,802 ± 1127†	592 ± 97

*Data are presented as mean ± SEM. pg/mL

†Significant at ≥ 95%. See text for p values.

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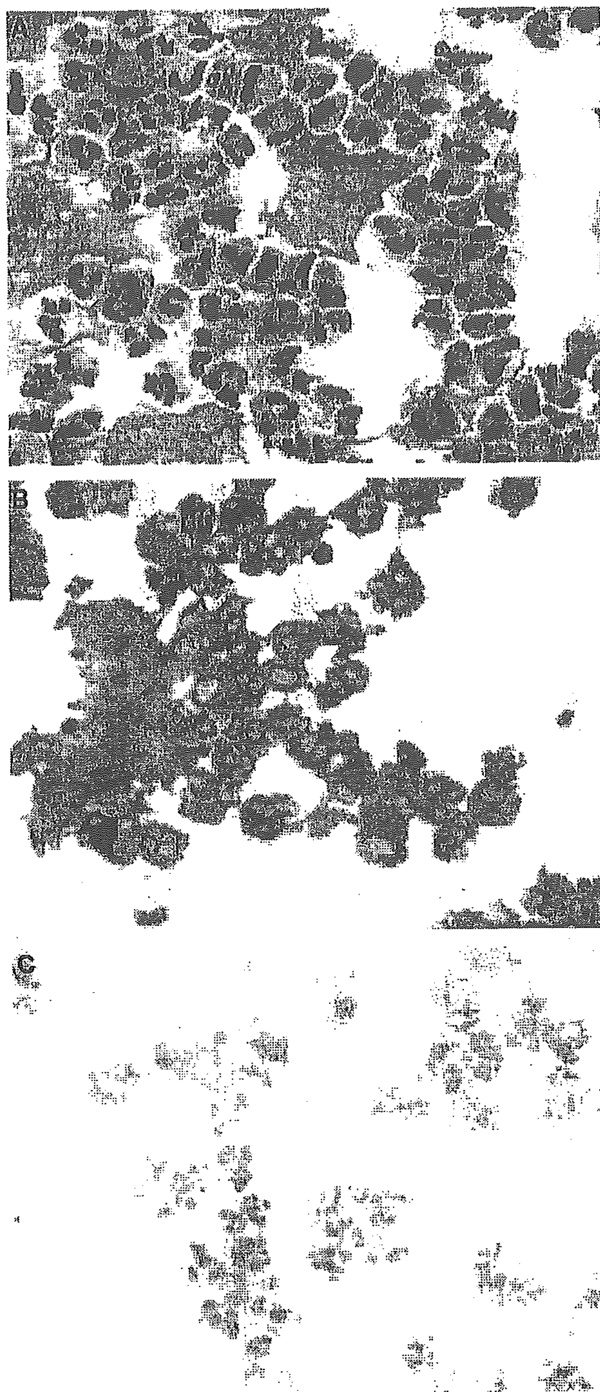

FIGURE 1. *Top*: Wright-Giemsa stain (original × 200) of induced-sputum sample from a CF patient showing neutrophil predominance. *Middle*: CF specimen stained for presence of CAP18 showing neutrophil granule localization. *Bottom*: isotype-negative control antibody result.

confirming the importance of neutrophil specific granules as a major source of airway CAP18 in CF (Fig 2).

Another neutrophil product important in CF pathobiology, IL-8, was also identified by flow cytometry in sputum cells that expressed CD15 (Fig 3). Similarly, PAI-1 was found, albeit at lower levels of expression, in CD15+ sputum cells (Fig 4).

Sputum levels of innate immune factors were correlated with each other in order to examine potential relationships in production or regulation. As shown in Table 2, levels of the three plasmin activation system components correlated with each other ($p < 0.0001$) but also with several cytokine responses (*eg*, uPAR and uPA with IL-8, $p < 0.0001$; uPAR and uPA with TNF-α, $p \leq 0.002$; and uPAR with IL-10, $p = 0.006$). In addition, uPAR was weakly correlated with CAP18 ($p = 0.01$). Among cytokines, IL-8 levels correlated with TNF-α and IL-10 ($p < 0.0001$), and IL-10 with IFN-γ ($p = 0.001$). CAP18 levels were correlated not only weakly with uPAR but also weakly with IL-8 ($p = 0.04$) and strongly with IL-10 ($p < 0.0001$).

To examine the potential role of these factors in disease activity or progression, innate immune factors were correlated with level of pulmonary function as determined by spirometric assessment of airflow obstruction (Table 2). Among all study subjects, FEV_1 percentage of predicted showed a strong negative correlation with sputum CAP18 and PAI-1 levels ($p \leq 0.0009$) and a weak negative correlation with uPAR levels ($p = 0.01$; Table 2). The overall negative correlation between pulmonary function and CAP18 was due primarily to results in patients with CF ($r = -0.40$, $p = 0.06$) and to a slightly lesser extent COPD ($r = -0.19$, $p = 0.11$). A similar negative correlation trend was seen between FEV_1 percentage of predicted and IL-8 in patients with CF ($r = -0.39$, $p = 0.056$) and patients with COPD ($r = -0.21$, $p = 0.08$). However, when innate factors were correlated with each other in patients with CF, CAP18 and IL-8 did not correlate with each other; instead, CAP18 levels correlated strongly with IL-10 ($r = 0.78$, $p = 0.001$) and IFN-γ ($r = 0.59$, $p = 0.002$), suggesting that elevated CAP18 levels in CF may represent a counterinflammatory response along with IL-10 and IFN-γ (IL-10 correlated strongly with IFN-γ, $r = 0.58$, $p = 0.001$). In contrast, levels of IL-8, a known proinflammatory factor in CF and COPD that we confirmed correlated negatively with pulmonary function (see above), correlated strongly with levels of uPAR ($r = 0.59$, $p = 0.002$), PAI-1 ($r = 0.5$, $p = 0.009$), and TNF-α ($r = 0.5$, $p = 0.007$). TNF-α correlated strongly with uPA ($r = 0.65$, $p = 0.0004$).

DISCUSSION

Research[1–2,30–31] has focused on the role of deranged aspects of innate immunity in the pathobiol-

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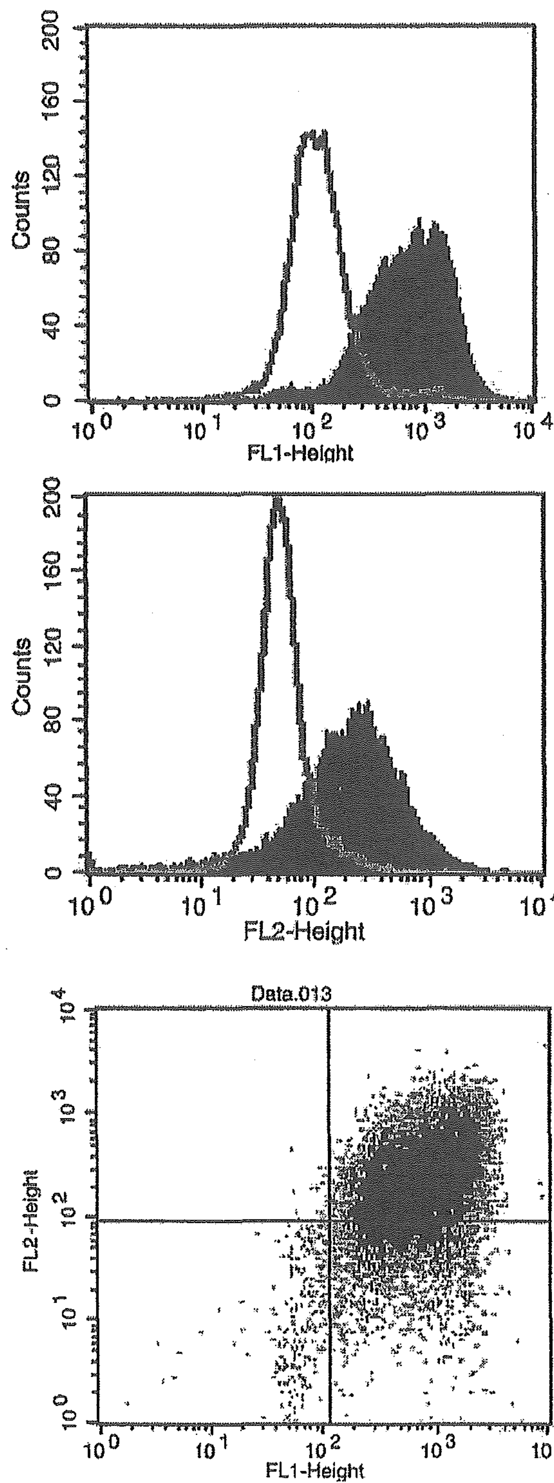

FIGURE 2. Flow cytometry for presence of CAP18 and CD15 in nonapoptotic (propidium iodide-negative) cells. *Top*: CF sample stained for CAP18 (purple) with isotype control antibody (open green) shown. *Middle*: same sample stained for CD15 with control. *Bottom*: two-color flow cytometry double-positive CAP18/CD15 cells are shown in upper right quadrant of gated cells (67.4% of total). Isotype control staining resulted in 1.0% double-positive cells (not shown). FL1 = fluorescence emissions wavelength 515 to 545 nm; FL2 = fluorescence emissions wavelength 564 to 606 nm.

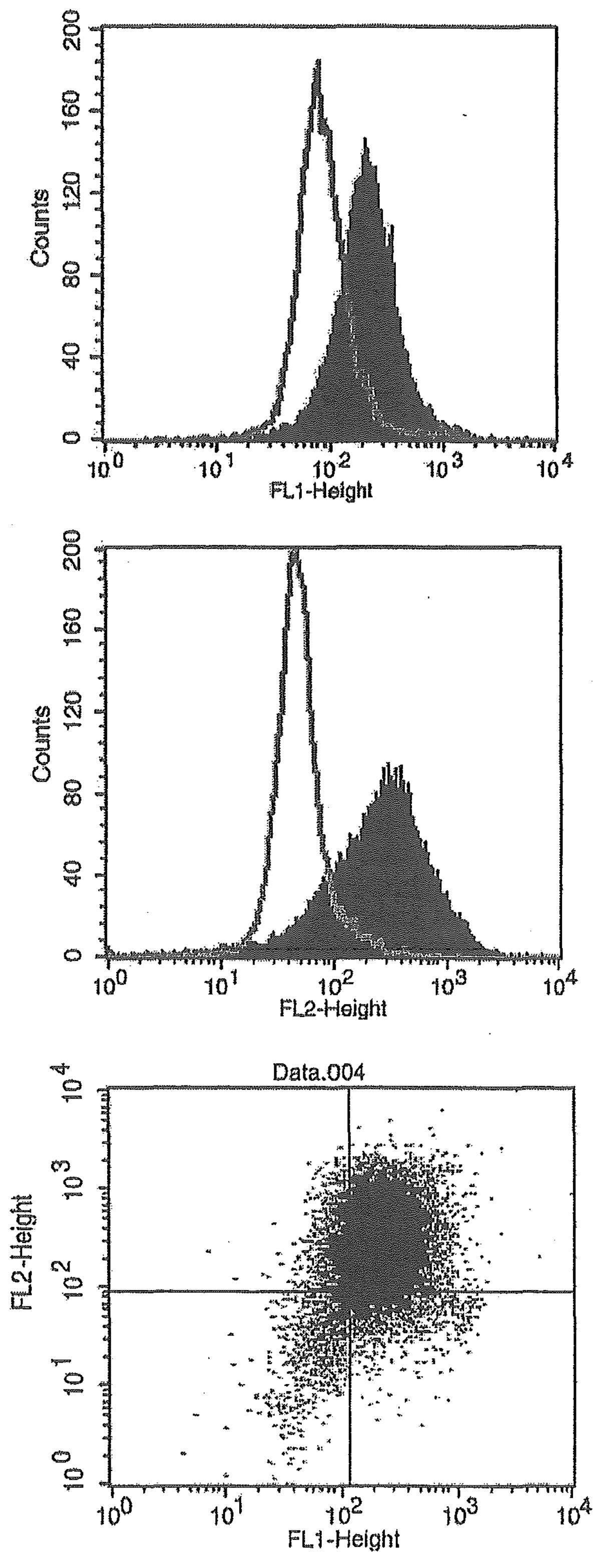

FIGURE 3. Flow cytometry for presence of IL-8 and CD15. *Top*: CF sample stained for IL-8 with isotype control shown as for Figure 2. *Middle*: same sample stained with CD15 and control. *Bottom*: double-positive IL-8/CD15 cells shown in upper right quadrant (64.4% of total). See Figure 2 legend for expansion of abbreviations.

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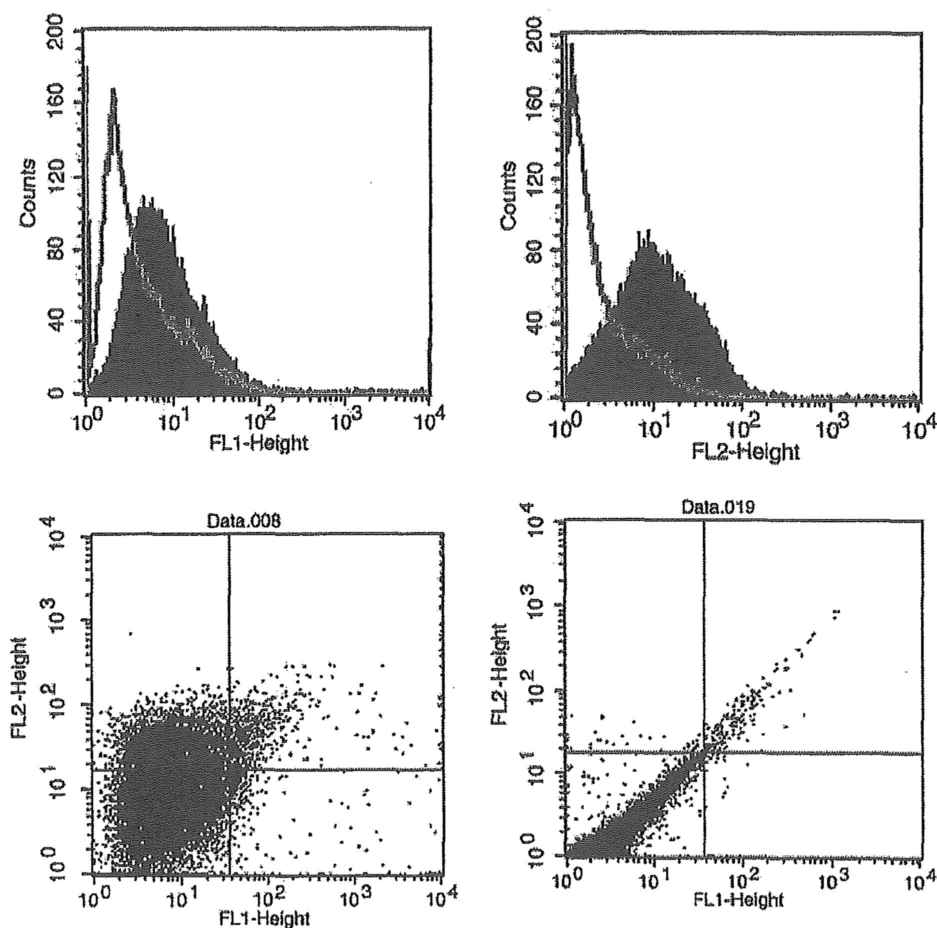

FIGURE 4. Flow cytometry for presence of PAI-1 and CD15. *Top left*: CF sample stained for PAI-1 with isotype control shown. *Top right*: same sample stained for CD15 and control. *Bottom left*: double-positive cells (upper right quadrant) representing 4.16% of total gated cells. *Bottom right*: double-positive cells in upper right quadrant (1% of total) using isotype control antibodies. See Figure 2 legend for expansion of abbreviations.

ogy of chronic inflammatory airways diseases such as CF, asthma, and COPD. In the present study, we were interested in aspects of the innate immune response in CF, in particular the following: (1) the role of the uPA system and the antimicrobial cathelicidin CAP18 that have not received much attention, and (2) how any abnormalities in CF compared to COPD and asthma as well as healthy persons. In order to study this local response noninvasively, we employed a standardized, validated sputum-induction protocol.[29]

The pulmonary plasminogen activator system involves multiple components including uPA, uPAR, and PAI-1. A saturable, specific binding of uPA to uPAR has been demonstrated in a number of cell types including monocytes, macrophages, mast cells, lymphocytes, fibroblasts, endothelial cells, and pulmonary airway epithelial cells.[32–33] Mature uPAR lacks the hydrophobic transmembrane and intracellular domains. Instead, a glycosylphosphatidylinositol moiety is added to the C-terminus providing the anchorage to the outer leaflet of the plasma membrane. Soluble uPAR has been isolated from normal and diseased plasma. By interacting with cell surface adapter molecules and extracellular proteins, uPAR acts beyond the traditional role of localizing and activating cell surface uPA and extends its effects to cellular adhesion and signal transduction.[32] The expression of uPAR is enhanced in various tumors and by a number of proinflammatory agents such as growth factors and cytokines.[32,34] Intraperitoneal injection of endotoxin to mice increased the expression of uPAR in many tissues.[35] Both membrane and soluble forms of uPAR were up-regulated in monocyte cultures by several bacterial surface proteins.[34] There is thus reason to suspect that chronic airways infection, such as seen in CF and COPD, may up-regulate uPAR, and indeed we found increased

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Table 2—*Significant Correlations of Induced Sputum Innate Immune Factors With Each Other and With Pulmonary Function Among All Study Subjects*

Factors	Correlates	r	p Value
CAP18	IL-10	0.53	0.0001
CAP18	IL-8	0.16	0.04
uPA	uPAR	0.61	0.0001
uPA	PAI-1	0.40	0.0001
uPA	IL-8	0.34	0.0001
uPA	TNF-α	0.24	0.001
uPAR	PAI-1	0.58	0.0001
uPAR	IL-8	0.47	0.0001
uPAR	TNF-α	0.22	0.002
uPAR	IL-10	0.20	0.006
uPAR	CAP18	0.19	0.01
PAI-1	IL-8	0.53	0.0001
IL-8	TNF-α	0.31	0.0001
IL-8	IL-10	0.30	0.0001
IL-10	IFN-γ	0.24	0.001
FEV_1	CAP18	− 0.31	0.0001
FEV_1	PAI-1	− 0.26	0.0009
FEV_1	uPAR	− 0.20	0.01

levels in sputum in CF and COPD, and to lesser degree in asthma, compared to normal. Interestingly, local uPA itself does not appear to be elevated in any of the disease groups, but its effects could be augmented by the up-regulated receptor levels seen.

PAI-1, a serine protease inhibitor belonging to the serpin family, is a single-chain 45- to 50-kd glycoprotein secreted by many cell types.[36–37] It binds to uPA to modulate the activity of uPA beyond plasmin activation to involve cell adhesion and tissue remodeling. PAI-1 may be inactivated by binding to uPA or forming complexes with uPA and uPAR that are internalized and digested in lysosomes. PAI-1 is secreted by many cell lines, and its expression can be regulated by hormones, growth factors, cytokines, and endotoxin in cell cultures.[37] Depending on the presence of specific regulatory agents, the expression of PAI-1 can either be enhanced or reduced. We found comparably increased levels of PAI-1 in all three chronic inflammatory airways diseases studied (Table 3). Marshall and Shute[38] reported quantitatively similar elevations of PAI-1 in CF sputum (mean, 5.7 ng/mL vs 1.0 ng/mL in control subjects, as compared with our mean of 3.8 ng/mL vs 0.6 ng/mL). During the inflammatory processes, autocrine and paracrine chemokine and cytokine secretion by neutrophils, monocytes, lymphocytes, and airway epithelial cells is enhanced. This may result in increased PAI-1 in sputum by different paths in these diseases.

Table 3—*Schematic Representation of Disease-Specific Patterns of Innate Immune Factors in Sputum as Compared to Healthy Control Subjects**

Factors	CF	COPD	Asthma
CAP18	↑	↑	↓
IL-8	↑↑	↑	↑
IL-10	↑	⟷	⟷
INF-γ	⟷	⟷	⟷
TNF-α	↑	⟷	⟷
uPA	⟷	⟷	⟷
uPAR	↑↑	↑↑	↑
PAI-1	↑	↑	↑

*↑ = elevated; ↓ = depressed; ↔ = equivalent.

Increases of the neutrophil chemotactic cytokine IL-8 level in airways of CF and COPD patients have been previously reported and related to pathogenesis.[4,28,39] IL-8 is produced by a variety of cells including neutrophils, monocytes, T-cells, and endothelial and airway epithelial cells.[39–42] IL-8 and uPAR both are chemotactic with monocyte and airway epithelial cell expression induced by bacterial products.[34,43] The interaction between leukocytes and endothelial cells is regulated by IL-8 via changes in integrin expression that also involve uPAR.[44–47] Therefore, integrins are potential mediators connecting the functions of IL-8 and uPAR (sputum levels of which we found to be highly correlated). Marshall et al[48] have also related IL-8 activity to PAI-1 by demonstrating that PAI-1 enhances IL-8 activity via inhibition of shedding IL-8/heparan sulfate/syndacan-1 complexes from endothelium. Indeed, IL-8 levels were highly correlated in our study not only with uPAR but also with PAI-1, suggesting a coordinate response in these diseases. The plasminogen activator system may play a prominent role in sustaining airway inflammation by increasing β_2-integrin–mediated leukocyte adhesion and also leukocyte adhesion to extracellular matrix vitronectin.[49–51]

With regard to cathelicidin CAP18, we found comparably elevated levels in CF and COPD patients and subnormal levels in asthmatics. These differences were due to local production, as systemic CAP18 levels in CF patients and control subjects were similar to each other and previously reported plasma levels (approximately 1 μg/mL).[52] CAP18 levels inversely correlated with pulmonary function, a relationship seen most strongly in CF patients but also in COPD. These changes have not been previously reported, although recently Chen et al[53] found increased CAP18 in BAL fluid from patients with CF; CAP18 levels correlated with neutrophilia and decreased lung function. Under normal conditions, CAP18 is secreted by airway epithelial cells and alveolar macrophages, but in conditions of neutrophilia this cell type would be expected to be the dominant source, as we found. The antimicrobial characteristics of CAP18 are related to its α-helical

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structure; positive ions, pH, and its own concentration affect the structure and change its antimicrobial activity.[54] It is unknown how changes in pH, ion concentration, sputum viscosity, and other factors in patients with chronic airways disease may affect the bioactivity of CAP18. We found that increases in CAP18 correlated with IL-10 and IFN-γ rather than IL-8 or TNF-α levels in CF, suggesting a possibly compensatory antiinflammatory rather than proinflammatory role. However, high concentrations of CAP18 may be cytotoxic to eukaryotic cells, and a deleterious effect certainly cannot be excluded.[54]

Sputum levels of PAI-1 also correlated negatively with pulmonary function. This suggests a relationship between PAI-1 and the degree of inflammation and tissue remodeling. PAI-1 binds uPA/uPAR, forming uPA/uPAR/PAI-1 complexes that are internalized across the cell membrane together with low-density lipoprotein receptor-related protein and degraded in the lysosome. uPAR is recycled to the cell membrane. PAI-1 thus not only controls the proteolytic activity of uPA but also modulates the number of uPAR on the cell surface.[15,55] The parallel increase of PAI-1 with uPAR suggests a finely tuned proteolytic balance is critical in the airway plasminogen activator system.[56] The dissolution and remodeling of extracellular matrix depends on a tightly controlled dynamic that maintains a proper balance between uPA/uPAR and PAI-1.[57] The increase in the PAI-1 may be homeostatic for the proproteolytic activity of increased uPAR.

In conclusion, comparison of CF to COPD and asthma as well as normal control subjects revealed interesting differences in innate immune factor levels. CAP18 is elevated in mild CF as well as COPD and inversely related to lung function, but correlation to IL-10 and IFN-γ suggests it may homeostatic rather than proinflammatory. The low levels of CAP18 in asthma are unexpected, perhaps reflecting the eosinophilic character of asthmatic inflammation or metabolic differences. Elevations of IL-8 in CF and COPD confirm prior studies, while levels of IL-10 have been variously reported to be low, normal, or elevated. TNF-α is only modestly increased in CF sputum, and IFN-γ levels are normal. CF and COPD, as noted above, share a common elevation of uPAR that is greater than that seen in asthma, while all three diseases show comparable increases in PAI-1. Neutrophils seem to be a prominent source of CAP18, IL-8, and PAI-1 in these diseases, These differing patterns allow may allow disease differentiation from a pathobiologic perspective and offer avenues for further research on pathogenetic pathways. For example, the striking differences observed in sputum CAP18 (elevated in CF and COPD, depressed in asthma) suggest further studies on the role of cathelicidins in specific forms of airway inflammation. The practical implications of such studies will likely emerge only after considerably more investigation.

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Sputum Cathelicidin, Urokinase Plasminogen Activation System Components, and Cytokines Discriminate Cystic Fibrosis, COPD, and Asthma Inflammation

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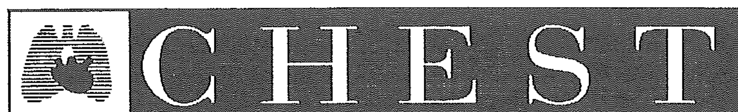

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Multidrug-Resistant *Pseudomonas aeruginosa* Strain That Caused an Outbreak in a Neurosurgery Ward and Its *aac(6')-Iae* Gene Cassette Encoding a Novel Aminoglycoside Acetyltransferase

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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

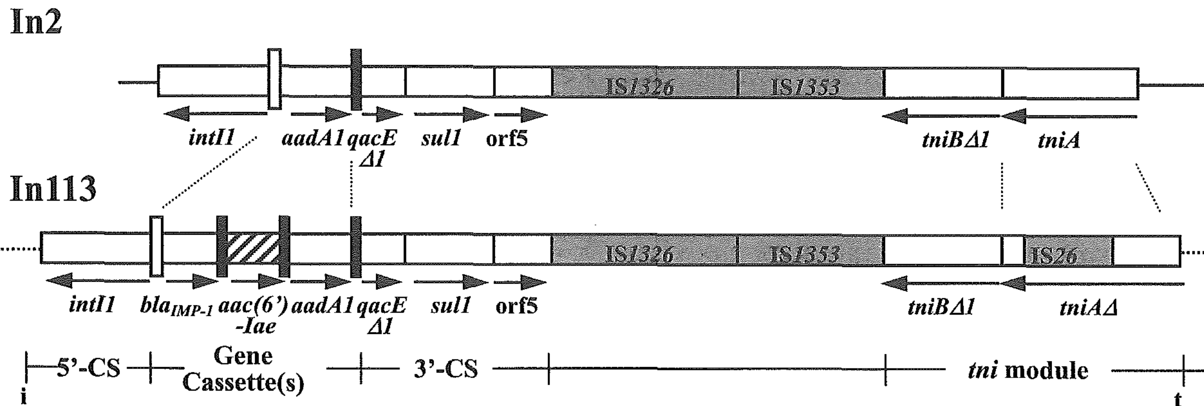

FIG. 1. Structure of In2 (GenBank accession no. AF071413) and In113. Gene cassettes are represented as open boxes with an adjacent vertical bar (59-be), shown as heavy solid vertical bars. The novel ORF found in In113 is shown as a hatched box. Genes are indicated by horizontal arrows. IS are represented 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*, *sul1*, 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-sul1* in a 4.7-kbp region, *sul1-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

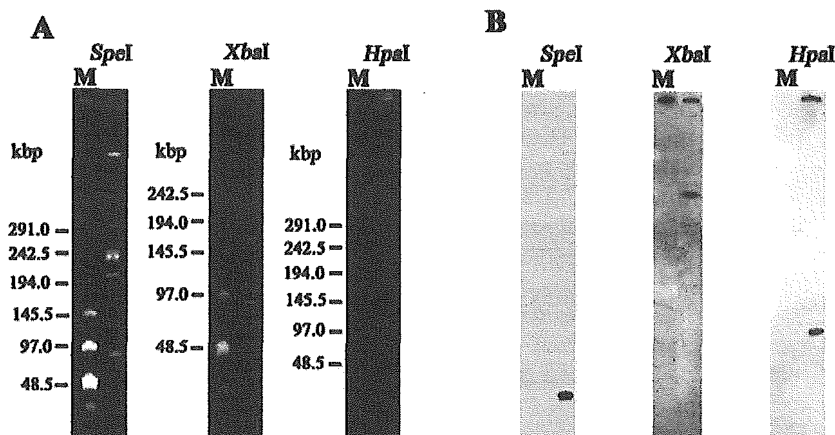

FIG. 2. (A) PFGE of SpeI-, XbaI-, and HpaI-digested genomic DNA from multidrug-resistant *P. aeruginosa* IMCJ2.S1. (B) Southern blotting of the same gels with an *aac(6′)-Iae* probe. Lanes M, HindIII-digested λ phage DNA as a size marker.

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

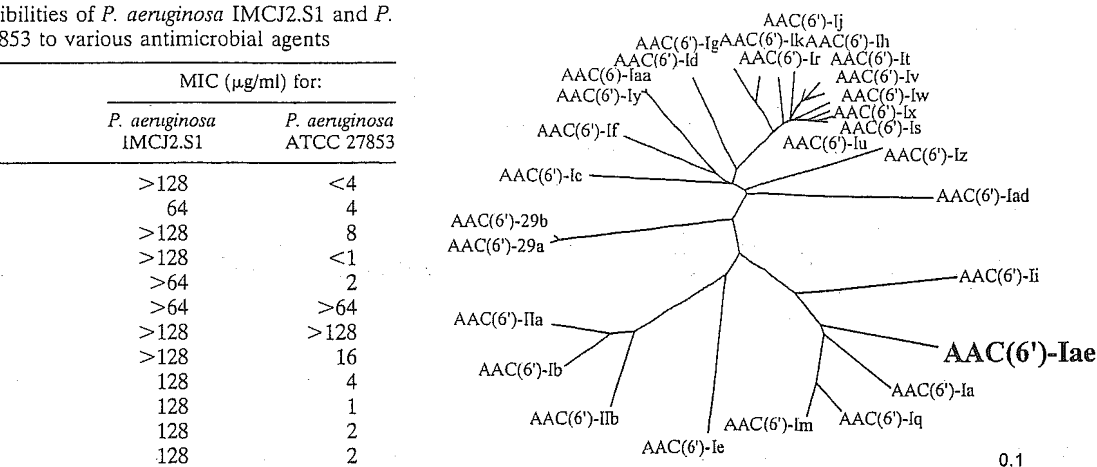

FIG. 4. Dendrogram of aminoglycoside 6′-*N*-acetyltransferases for comparison with AAC(6′)-Iae. The dendrogram was calculated with the CLUSTAL W program. Branch lengths correspond to the number of amino acid exchanges for AAC proteins. EMBL/GenBank/DDBJ accession numbers of AAC proteins are as follows: 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

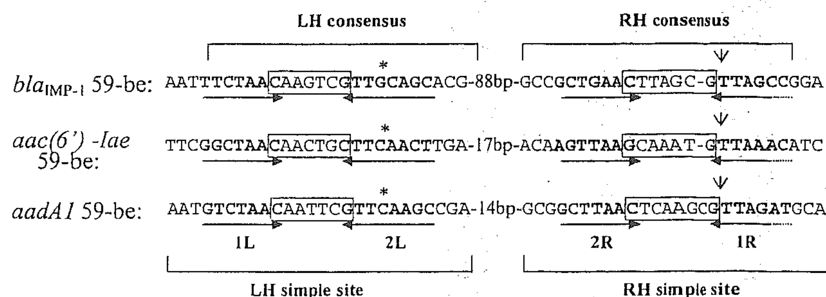

FIG. 3. Structures of 59-be of In113. Seven-base-pair putative core sites in the left-hand (LH) and right-hand (RH) consensus sequences were designated 1L and 2L and 2R and 1R, respectively. The putative recombination event occurs between the G and the first T in the 1R core site and is indicated by 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.

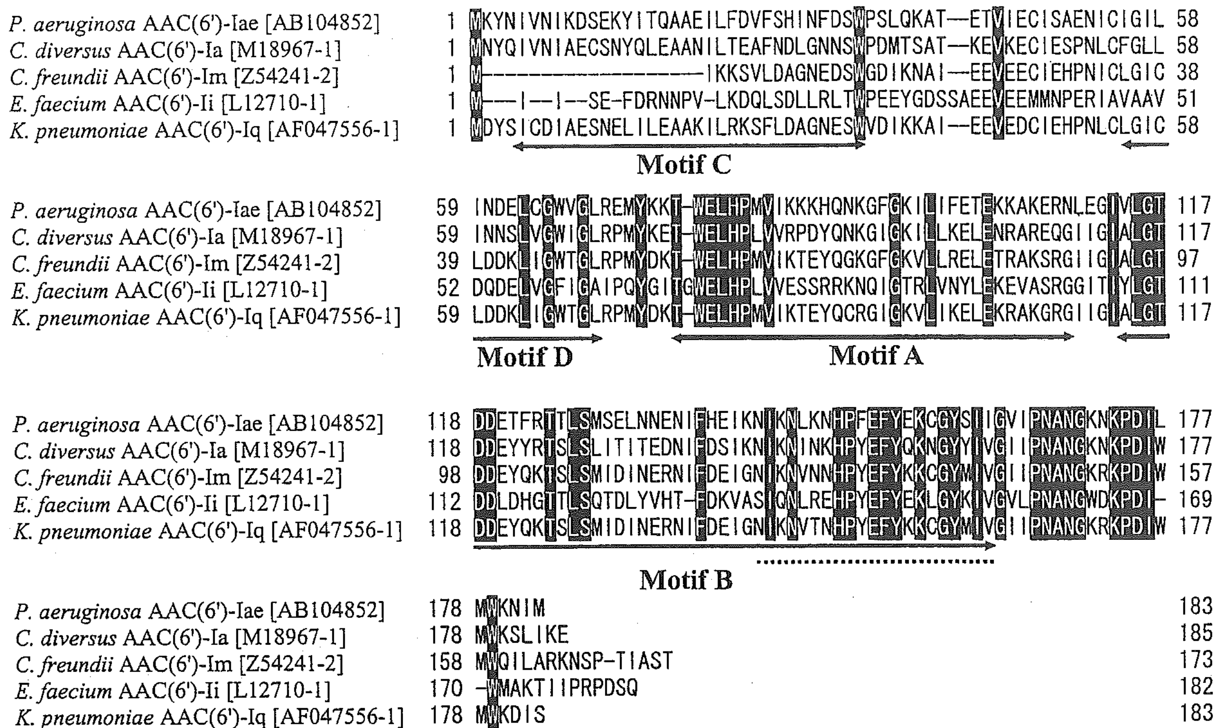

FIG. 5. Alignment of the AAC(6')-Iae amino acid sequence with those of four members of the AAC(6')-I subfamily. Identical residues are marked with black boxes. Four motifs, including the highly conserved motif B, are underlined. A conserved region of 21 amino acids, described by Shmara et al. (44), is 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).

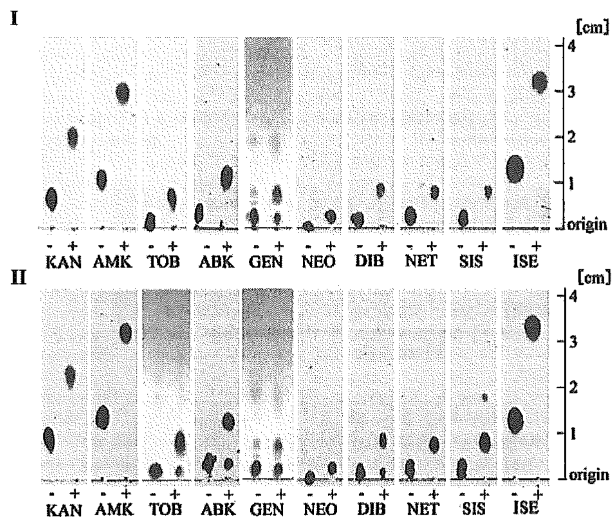

FIG. 6. Thin-layer chromatogram of aminoglycosides incubated with AAC(6′)-Iae protein (I) or with AAC(6′) from *Streptomyces lividans* TK21 as a control (II) (53) in the presence (+) or absence (−) of acetyl coenzyme A. KAN, kanamycin; AMK, amikacin; TOB, tobramycin; ABK, arbekacin; GEN, gentamicin;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

```
                       TT             T                                  T
                      T NN       NN   N N    TT  T                     NNN    T    T            NNN N
S3DHFR          ----MKVSLI AAMDKNRVIG KENDIPWRIP KDWEYVKNTT KGHPIILGRK NLESIG-RAL PDRRNIILTR DKGFTFNG-C
S2DHFR          MWSFLKISLI VAMDKKRVIG KDNDIPWRIS SDWEYVKNTT KGHAIILGRK NLQSIG-RAL PDRRNIILTR DKNFNFKD-C
B. anthracis    ----MIVSFM VAMDENRVIG KDNNLPWRLP SELQYVKKTT MGHPLIMGRK NYEAIG-RPL PGRRNIIVTR NEGYHVEG-C
B. cereus10987  ----MIVSFM VAMDENRVIG KDNNLPWRLP SELQYVKKTT MGHSLIMGRK NYEAIG-RPL PGRRNIIVTR NEGYHVEG-C
B. cereus14579  ----MIVSFM VAMDENRVIG KDNNLPWRLP SELQYVKKTT MGHPLIMGRK NYEAIG-RPL PGRRNIIVTR NEGYHVEG-C
B. subtilis     -----MISFI FAMDANRLIG KDNDLPWHLP NDLAYFKKIT SGHSIIMGRK TFESIG-RPL PNRKNIVVTS APDSEFQG-C
E. faecalis     -----MLAAI WAQDEQGVIG KEGKLPWHLP NDLKFFKEKT IHNTLVLGRA TFEGMGCRPL PNRTTIVLTS NPDYQAEG-V
E. faecium      -----MFISM WAQDKNGLIG KDGLLPWRLP NDMRFFREHT MDKILVMGRK TYEGMGKLSL PYRHIIVLTT QKDFKVEKNA
S. pneumoniae   --MTKKIVAI WAQDEEGVIG KENRLPWHLP AELQHFKETT LNHAILMGRV TFDGMGRRLL PKRETLILTR NPEEKIDG-V
S1DHFR          ----MTLSII VAHDKQRVIG YQNQLPWHLP NDLKHIKQLT TGNTLVMARK TFNSIG-KPL PNRRNVVLTN QASFHHEG-V
SeDHFR          ----MTLSII VAHDKQRVIG YQNQLPWHLP NDLKHVKQLT TGNTLVMGRK TFNSIG-KPL PNRRNVVLTN QASFHHEG-V
SaDHFR          ----MTLSIL VAHDLQRVIG FENQLPWHLP NDLKHVKKLS TGHTLVMGRK TFESIG-KPL PNRRNVVLTS DTSFNVEG-V
E.coli K-12     -----MISLI AALAVDRVIG MENAMPWNLP ADLAWFKRNT LNKPVIMGRH TWESIG-RPL PGRKNIILSS- SQPGTDDR-V

                                         NNN NNN                T          NN
S3DHFR          EIVHSIEDVF ELCKN-EEEI FIFGGEQIYN LFFPYVEKMY ITKIHHEFEG DTFFP-EVNY EEWNEVFAQK GIKNDKNPYN
S2DHFR          EIAHSIEAAF KLCEN-EEEV FIFGGEQIYV MFLPYVEKMY VTKIHHEFEG DTFFP-VVNF DDWKEVSVEK GIKDEKNPYD
B. anthracis    EVAHSVEEVF ELCKN-EEEI FIFGGAQIYD LFLPYVDKLY ITKIHHAFEG DTFFP-EMDM TNWKEVEFVEK GLTDEKNPYT
B. cereus10987  EVAHSVEEVF ELCKN-EEEI FIFGGAQIYD LFLPYVDKLY ITKIHHAFEG DTFFP-EMDM TNWKEIFVEK GLTDEKNPYT
B. cereus14579  EVVHSVEEVF ELCKN-EEEI FIFGGAQIYD LFLPYVDKLY ITKIHHAFEG DTFFP-EIDM TNWKEIFVEK GLTDEKNPYT
B. subtilis     TVVSSLKDVL DICSG-PEEC FVIGGAQLYT DLFPYADRLY MTKIHHEFEG DRHFP-EFDE SNWKLVSSEQ GTKDEKNPYD
E. faecalis     LVMHSVEEIL AYADKYEGVT VIGGGSVVFK ELIPACDVLY RTMIXETFEG DTFFP-EIDW SVWEKVATVP GVVDEKNLYA
E. faecium      EVLHSIDELL AYAKDIPEDI YVSGGSRIFQ ALLPETKIIW RTLIDAEFEG DTFIG-EIDF TSFELVEEHE GIVNQENQYP
S. pneumoniae   ATFQDVQSVL DWYQDQEKNL YIIGGKQIFQ AFEPYLDEVI VTHIHARVEG DTYFPEELDL SLFETVSSKF YAKDEKNPYD
S1DHFR          DVINSLDEIK ELSGH----V FIFGGQTLYE AMIDQVDDMY ITVIDGKFQG DTFFP-PYTF ENWEVESSVE GQLDEKNTIP
SeDHFR          DVINSLDEIK ELSGH----V FIFGGQTLFE AMIDQVDDMY ITVIDGKFQG DTFFP-PYTF ENWEVESSVE GQLDEKNTIP
SaDHFR          DVIHSIEDIY QLPGH----V FIFGGQTLFE EMIDKVDDMY ITVIEGKFRG DTFFP-PYTF EDWEVASSVE GKLDEKNTIP
E.coli K-12     TWVKSVDEAI AACGD-VPEI MVIGGGRVYE QFLPKAQKLY LTHIDAEVEG DTHFP-DYEP DDWESVFSEF HDADAQNSHS

S3DHFR          YYFHVYERKN LLS----  (Accession number AB205645)
S2DHFR          YYFHIYERIR -------  (Accession number Z50141-2)
B. anthracis    YYYHVYEKQQ -------  (Accession number AE017031-55)
B. cereus10987  YYYHVYEKRQ -------  (Accession number AE017271-169)
B. cereus14579  YYYHVYEKQQ -------  (Accession number AE017005-25)
B. subtilis     YEFLMYEKKN SSKVGGF  (Accession number L77246-26)
E. faecalis     HDYETYHRND K------  (Accession number AE016951-308)
E. faecium      HRFQKWQKMS KVV----  (Accession number 741860A)
S. pneumoniae   FTIQYRKRKE V------  (Accession number Z74778-1)
S1DHFR          HTFLHLVRRK GK-----  (Accession number X13290)
SeDHFR          HTFLHLVRRK GK-----  (Accession number Z48233)
SaDHFR          HTFLHLIRKK -------  (Accession number Z16422)
E.coli K-12     YCFEILERR- -------  (Accession number P00379)
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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.