どをせずに患者の発症中又はその回復から10日以内の気道分泌物，体液，排泄物に直接触れたかその付着したものに直接触れた者，(3)患者と2メートル以内で会話をした者が濃厚な暴露を受けた者としている。待合室や職場での短時間一緒に過ごした，歩いてすれ違ったなどの場合は入らない。

4）飛沫核：飛沫から水分が蒸発したり上皮細胞が脱落して直径が5μm以下になった小さな粒子で落下速度は0.06～1.5cm/secと非常に遅く，空中に長く浮遊し，風により遠くまで，時に100m以上の遠方まで飛散する。

5）空気感染：飛沫核の吸入により感染が伝播する経路。肺結核，麻疹，水泡などがある。SARSでは証明されていない。

・潜伏期間

多くは2～7日間。最大10日間以内と考えられている。

・感染期間

潜伏期あるいは無症状期における他への感染力はない，あったとしても極めて弱いと考えられている。前駆期に相当する発熱・咳嗽期の患者は，感染力は弱いが，十分な警戒が必要である。肺炎の極期や重傷者ほど感染力が強い。

・症状

およそ2～7日（最大10日）の潜伏期間を経過した後，38度以上の急な発熱で発病し，咳，全身倦怠，筋肉痛などのインフルエンザ様の前駆症状が現れる。2～数日間で呼吸困難，乾性咳嗽，低酸素血症などの下気道炎症が現れ，胸部CT，X線写真などで肺炎像が出現する。肺炎になった者の80～90%が1週間程度で回復傾向になるが，10～20%が人口呼吸器などを必要とするほど重症となる。致死率は10%前後で，24歳以下1%の死亡率，25～44歳で6%，45～64歳で15%，65歳以上で50%以上。重症化の要因としては，高齢，糖尿病，B型肝炎，慢性肝炎などの基礎疾患，喫煙。

・患者の判断基準

図4に示す。

図4　SARS疑い例，疑似症例，患者の判断基準

※いずれの場合においても，他の診断によって症状が説明できる場合は除外するものとする。

SARS疑い例とは

平成14年11月1日以降（遡及報告も含む）に，38度以上の急な発熱及びせき，呼吸困難等の呼吸器症状を示して受診した者，または病理解剖の行われていない死亡者のうち，以下のいずれかを満たす者

① 発症前10日以内に，WHOが公表したSARSの伝播確認地域へ旅行した又は居住していた者

② 発症前10日以内にSARSが疑われる患者を看護・介護するか，同居しているか，気道分泌物，体液に直接触れた者

SARS疑似症例とは

SARS疑い例のうち，以下のいずれかを満たす者

①胸部X線で肺炎，又は呼吸窮迫症候群の所見を示す者

②病理解剖所見が呼吸窮迫症候群の病理所見として矛盾せず，はっきりとした原因のない者

SARS患者（確定例）とは

症状や所見から当該疾患が疑われ，病原体診断や血清学的診断がなされた者

＜材　料＞咽頭ぬぐい液，喀たん，尿，便，血液

＜診断法＞病原体検出（ウイルス培養），遺伝子検出（RT－PCR），抗体検出（ELISA等）

図5　SARS診断のまとめ

	最近10日以内の流行地へ旅行あるいは在住	38℃を越える発熱	咳，呼吸困難などの呼吸器症状	胸部X線検査で肺炎像	ウイルス検査又は血清抗体検査	保健所への届出
SARS	あり	あり	あり	あり	陽性	1類*として届出
擬似SARS（従来の可能性例にほぼ同じ）	あり	あり	あり	あり	陰性又は未検査	1類として届出
SARS疑い例	あり	あり	あり	異常なし		保健所に報告
一般呼吸器疾患	なし	あり又はなし	あり	あり又は異常なし		必要なし

6）感染症法（「感染症の予防および感染症の患者に対する医療に関する法律」）は，"感染症の予防および感染症の患者に対する医療に関し，必要な処置を定めることにより，感染症の発症を予防し，およびその蔓延の防止を図り，もって公衆衛生の向上および増進を図ること"を目的に1999年に施行された。その後，海外における感染症の発生状況，国際交流の進展による人や物の移動の活発化および迅速化，保健医療を取り巻く環境の変化に伴う感染症対策の充実の要請に応えて2003年11月5日に改正が行われた。感染の重篤度に応じて1類から5類まで分類され，SARSはコロナウイルスという病因がわかったことでもっともシビアな疾患群として1類感染症に分類された（図5）。

・病原体

原因ウイルスは，新型のコロナウイルスで，SARSコロナウイルスである（図6）。エンベロープというウイルス粒子の一番外側にある膜のあるウイルスである。この膜は脂質2重層に，糖タンパクが挿入された構造をとる。消毒剤を作用させたときこの膜のあるウイルスの方が膜のないウイルスよりも消毒剤で感染力がなくなりやすい。SARSコロナウイルスは，乾燥

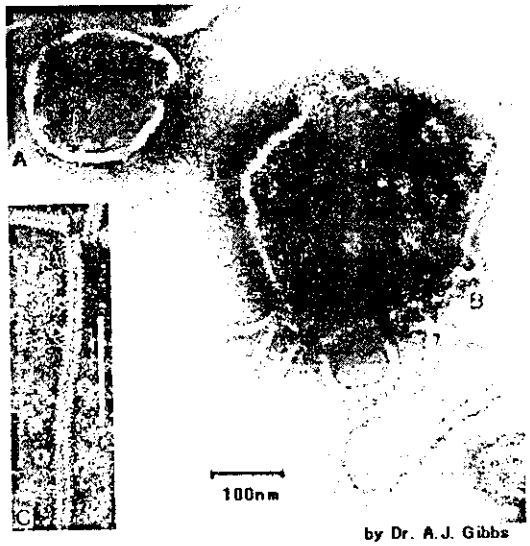

図6　SARSコロナウイルスの電子顕微鏡像
原因ウイルスは，新型のコロナウイルスで，SARSコロナウイルスである。エンベロープというウイルス粒子の一番外側にある膜のあるウイルスである。

したプラスチックの上で48時間生存したという報告がある。また，下痢便中で4日間，尿中で24時間生存したという報告もある。鼻咽頭拭い液や排泄物中のウイルス分泌期間は図7のとおりである。鼻咽頭拭い液には発症初期からSARSウイルスが分泌され，3～5日には糞便

病日	0－2日	3－5日	6－14日	15－17日	21－23日
鼻咽頭拭い液	31％	43%	57－69%	35%	13%
糞便	0%	57%	86－100%	33%	43%
尿			50%	34%	21%

図7　鼻咽頭拭い液や排泄物中のウイルス分泌期間
鼻咽頭拭い液には発症初期からSARSウイルスが分泌され，3～5日には糞便中にも分泌されるようになり，尿中含めて6～14日にピークに達する。

中にも分泌されるようになり，尿中含めて6～14日にピークに達する。現在，この新型のコロナウイルスのさらなる研究が進められている段階であるが，有効なワクチンはまだできていない。

・SARSの治療

対症療法が中心となる。種々の治療が試みられているが，コンセンサスを得られている有効な方法はない。

・SARSコロナウイルスの消毒

(1)　加熱滅菌可能なもの

高圧蒸気(オートクレーブ)滅菌(121℃，20分)，乾燥滅菌（180～200℃，1時間あるいは160～170℃，2時間)，煮沸消毒（98℃以上，15分以上)

(2)　加熱滅菌不可能なもの

現在のところ，その効果と入手の容易さなどから，消毒用エタノール及び界面活性剤の使用が推奨される。基本的に消毒剤の噴霧は避け，広い面などでは拭き取り，可能なものについては消毒剤へ漬け置きすることも検討する。消毒剤が触れている時間が長いほうがより効果が高い。

a）消毒用エタノール（70～80%）

SARSコロナウイルスに有効である。人体に対する毒性がなく，手指の消毒などに適している。脱脂効果のため皮膚が荒れることがあるので，スキンケアが重要である。血液が付着している場合などには，内部まで届かないことがあり洗い落とす必要がある。引火性，揮発性があるので取り扱いに注意が必要。

b）界面活性剤

従来のコロナウイルス及びSARSコロナウイルスに対して有効性が確認されている。(国立感染症研究所未発表データ）効果が確認されているのは食器・野菜洗浄用の家庭用合成洗剤であり，成分として直鎖アルキルベンゼンスルホン酸ナトリウムもしくはアルキルエーテル硫酸エステルナトリウムを16%以上含むものである。家庭用合成洗剤における界面活性剤の濃度は製品により異なるが，SARSコロナウイルスの消毒として推奨される「台所用合成洗剤をぬるま湯1リットルに対し5～10ml程度加えたもの」。

c）過酢酸

低濃度（0.001～0.2%）で芽胞を含むすべての微生物に対して有効である。もちろんSARSに対しても有効である。

d）グルタルアルデヒド（2%，pH8）

化学作用，蛋白質変性作用が強く，殺菌力も強いためあらゆる微生物を消毒することが可能である。刺激が強いために人体へは使用できない。器具の消毒には血液体液を十分に除去した後，2%グルタルアルデヒド溶液に1時間浸漬の後，十分に水洗する。排泄物や体液の消毒には2時間以上浸漬するほうが確実である。

e）次亜塩素酸ナトリウム

有効塩素濃度は，0.02%～0.05%（200－500ppm）で1時間以上浸漬使用するこ

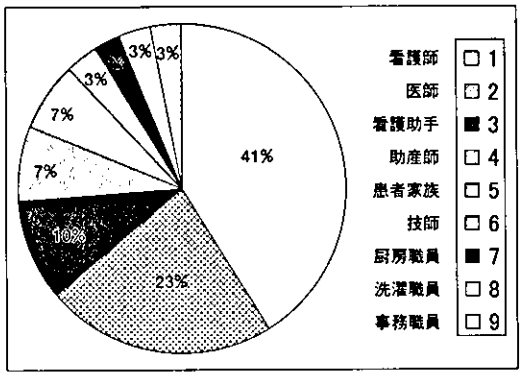

図8　ハノイフレンチ病院に入院したSARS患者の内訳

ベトナムハノイフレンチ病院に入院したSARS患者39人の内訳を示す。80%以上が，看護師，医師，看護助手，助産師などの医療従事者だった。

組織性	病因	特徴
咽頭炎	*Streptococcus pyogenes*	咽頭の炎症：咳，鼻汁(-)発熱
咽頭炎および咽頭頭蓋炎	*Haemophilus influenzae*	咽頭および咽頭蓋の炎症
副鼻腔炎	*Streptococcus pneumoniae* *H. influenzae S. pneumoniae* *Staphylococcua aureus S. pyogenes*	副鼻腔の炎症，ときどき激痛
気管支炎	*S. pneumoniae* *Mycoplasmapneumoniae*	粘液膿が混じる咳を伴う気管と細気管支の炎症
ジフテリア	*Corynebacterium diphtheriae*	偽膜および全身性毒素を伴う咽頭の炎症
外耳炎	*S. aureus* *Pseudomonas aeruginosa*	外耳道の炎症
中耳炎	*S. pneumoniae S. pyogenes*	圧と痛みを伴う膿様滲出物の感染症
ウイルス性		
普通感冒	Rhinoviruse, Coronaviruses	咽頭痛，倦怠，頭痛，咳
パラインフルエンザ	Parainfluenza virus	鼻炎，咽頭痛，気管支炎，肺炎

図9　微生物感染による上気道疾患

SARS以外にも図のような微生物感染による上気道疾患があることも認識しておく必要がある。

とが多いが，確実な殺ウイルス作用を期待するためには0.1%（1000ppm）以上30分以上の作用が有効である。布，金属に対して腐食性があり，有機物が付着していると効果が低下する。人体には使用できない。合成洗剤入りの次亜鉛素酸ナトリウム製剤の方がSARSコロナウイルスにはより有効と考えられる。

f）塩化ベンザルコニウム，グルコン酸クロルヘキシジンともに手指の消毒の利用頻度が高いが，SARSコロナウイルスに対して効果が十分に得られない場合がある。

・院内感染予防対策

SARSは，患者の半数以上が医療従事者および訪問者であること，すなわち院内感染が重要なキーになっていることが特徴である。図8では，ベトナムハノイフレンチ病院に入院したSARS患者39人のの内訳を示す（川名明彦，感染症学会誌）。80%以上が，看護師，医師，看護助手，助産師などの医療従事者だった。歯科医院においても知らずに治療を行った場合，歯科医師，歯科衛生士が感染する確立は高いと考えられる。院内感染伝播を防ぐ方法は，すべての患者に対して行う標準予防策と感染経路別の予防策がある。またSARS以外にも微生物感染による上気道疾患があることも認識しておく必要がある（図9）。

標準予防策（スタンダードプリコーション[7]）：感染の有無に拘わらず，すべての患者に適用される感染予防策。血液，汗以外の体液，唾液などの分泌液及び排泄物，損傷した皮膚，及び粘膜，に適用される。歯科医療においては以下の具体的な項目があげられる。

(1) 手洗い
(2) 手袋
(3) マスク[8]
(4) メガネ
(5) 帽子
(6) 針刺し事故防止
(7) 医療器具の消毒
(8) 院内清掃
(9) 患者ごとのハンドピースの交換
(10) 他の患者に使用した麻酔カートリッジの完全廃棄
(11) スタッフの教育

飛沫感染予防策：歯科用ユニット間は1メートル以上離す。ユニット間にパーティションを設置する。可能なら個室管理。1メートル以内

の会話ではマスク着用。口外バキュームの設置。

接触感染予防策：患者の個室管理。手袋，防水エプロンの使用。衛生的手洗いの励行。診療器具の個別化。環境の消毒。特にドアノブも消毒。

7）感染対策は，感染の事実が判明している患者にだけ行うのではなく，すべての患者を感染症の可能性があるものとみなし，血液・体液を普遍的に排除しようとする考え方をユニバーサルプリコーションという。このユニバーサルプリコーションを一歩進めたボディ・サブスタンス・アイソレーションズ（Body Substance Isolations）が推奨され，さらにこの両者の長所を統合・調整したスタンダードプリコーション（Standard Precaution）へと改良された。歯科臨床における感染対策では，ユニバーサルプリコーションとスタンダードプリコーションに差がないため，歯科ではユニバーサルプリコーションと表現する場合が多い。歯科におけるユニバーサルプリコーションの対象物は，血液，分泌液，浸出液，組織，抜去歯牙およびこれらが付着したガーゼ，布，棉，紙などである。

8）口および鼻を被い，粒子の吸入や拡散を防ぐ用具。以下の4種類のマスクがある。

N95マスク：微粒粉塵の吸入防止する工業規格のマスク。空気感染防止の目的で使用する。通常の呼吸圧での規格のため咳やくしゃみのある人には使用してはならない。

サージカルマスク：通常マスクを手術などの医療行為に便利なように改良された人口繊維で出来た軽いマスク。鼻にあたる部分に銅線が入って，顔にぴたりと当たるようになる。

通常マスク：一般に市販されているガーゼマスク。柔らかいため顔にピタリとつく。ウイルスなど微粒子は通過可能であるが，多くは繊維により保着される。吸入する空気の加湿，保温に非常に有効で，気道粘膜の乾燥や微生物の付着・増殖から守るのに有効である。湿りやすい，少し重いのは他はサージカルマスクと効果は同等である。

簡易マスク：1枚の薄い紙にゴム線で耳にかけられるようにしたマスク。顔にピタリとつかず，鼻や口の周りに多くの隙間が出来やすい。

・歯科医院における院内感防止手順

1．管轄の保健所および地域の1類感染症指定病院の連絡先（日中，夜間および休祭日）を電話番号に貼っておく。

2．スタッフにSARSの教育およびスタンダードプリコーションのトレーニングを行う。

3．1）「SARSに関する注意」を施設の外（待合室に入る扉の外側など）の目立つ場所に貼っておく。＊SARSの流行が始まった場合。

SARSに関してのお知らせ

最近10日以内にSARS流行地へ旅行あるいは居住しておられた方で，38度を超える発熱または咳など呼吸器症状のある方は，来院せずに，保健所（電話番号）へご相談ください。

○○歯科医院

2）咳，クシャミ等に関するエチケットを受付や待合室に貼っておく。

かぜ症状の方へ

咳，クシャミ等かぜ症状のある方は，マスクを着用してください。マスクがない場合は，ハンカチかティシュで口元・鼻を覆いましょう。

○○歯科医院

4．受付にマスク，手洗い場にペーパータオルを備える。(共用タオルは置かない)

5．施設としての対応ステップ

平常時：スタッフ教育，マニュアル作成，インフルエンザワクチン接種推進，咳，発熱患者を診療する際は，医療職員はマスク着用，かぜ，インフルエンザシーズンの時は，外来待合室の患者にマスク着用を勧める。

世界のどこかでSARS発生：SARS情報収集，SARS関連ポスターの張り出し，電話対応。

日本国内でSARS発生：国内外の情報収集，院内感染対策の再強化を図る。

6．市民・患者からの電話問い合わせに対する対応

一般の歯科医院で対応すべきか，保健所へ相談すべきか。後の連絡のために，姓名，性，年齢および住所，連絡のとれる電話番号を聞いておく。

・SARSに関する最新情報の入手

国立感染症研究所感染症情報センター（http：//idsc.nih.go.jp/others/sars/index.html)，厚生労働省（http：//www.mhlw.go.jp)，海外渡航者のための感染症情報（http：//www.forth.go.jp/)，WHO (http://www.who.int/en/)，米国CDC(http：//www.cdc.gov/page.do）などのホームページなどから最新情報を得る際に参考となる。

さいごに

このような出筆する機会を与えていただいた東京都歯科医師会会員の皆様に深く感謝いたします。今後は，厚生省科学研究班を基にして，歯科医療における新規感染症に対する院内感染対策について検討を重ね充実したものに仕上げていこうと考えております。

【参考】

1）国立感染症研究所感染症情報センターホームページ (http：//idsc.nih.go.jp/others/sars/index.html)
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Original Article

Biofilm Formation among Methicillin-Resistant *Staphylococcus aureus* Isolates from Patients with Urinary Tract Infection

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Staphylococci have been confirmed to form biofilms on various biomaterials. The purpose of this study was to investigate biofilm formation among methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from patients with urinary tract infection (UTI) and to assess the relationship between biofilm-forming capacities and virulence determinants/clinical background. Over a 12-year period from 1990 through 2001, a total of 109 MRSA isolates were collected from patients (one isolate per patient) with UTI at the urology ward of Okayama University Hospital. We used the *in vitro* microtiter plate assay to quantify biofilm formation. We then investigated the presence of several virulence determinants by polymerase chain reaction assay and found eight determinants (*tst, sec, hla, hlb, fnbA, clfA, icaA, and agrII*) to be predominant among these isolates. Enhanced biofilm formation was confirmed in *hla-, hlb-, and fnbA*-positive MRSA isolates, both individually and in combination. Upon review of the associated medical records, we concluded that the biofilm-forming capacities of MRSA isolates from catheter-related cases were significantly greater than those from catheter-unrelated cases. The percentage of *hla-, hlb-*, and *fnbA*-positive isolates was higher among MRSA isolates from catheter-related cases than those from catheter-unrelated cases. Our studies suggest that MRSA colonization and infection of the urinary tract may be promoted by *hla, hlb*, and *fnbA* gene products.

Key words: methicillin-resistant *Staphylococcus aureus*, urinary tract infection, biofilm formation

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been identified as a major pathogen in nosocomial infections [1, 2]. The percentage of MRSA among nosocomial *S. aureus* isolates in Japan is estimated to be 50% to 70% [3]. The incidence of urinary tract infection (UTI) caused by MRSA is increasing because patients are more frequently fitted with various urinary stents and catheters as endourology progresses technologically [4].

Staphylococci, including *S. aureus*, are known to form biofilms on various biomaterials [5]. These organisms can persist in clinical settings and gain increased resistance to antimicrobial agents through biofilm formation that appears to be a bacterial survival strategy [6, 7]. Therefore, biofilms formed by MRSA have become resistant to most available antimicrobial agents. The polysaccharide intracellular adhesin (PIA), encoded by *ica* genes, has been shown to be required for biofilm

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formation by staphylococci [5]. More recently, α-toxin (Hla) has also been shown to play an integral role in biofilm formation [8]. The pathogenesis of *S. aureus* is attributed to the combined effects of extracellular factors and toxins, together with invasive properties such as adherence, biofilm formation, and resistance to phagocytosis.

S. aureus secretes a plethora of virulence factors such as toxins and enzymes [9], some of which cause particular diseases. For example, toxic shock syndrome toxin-1 (TSST-1) causes toxic shock syndrome (TSS) and staphylococcal enterotoxins (SEA, SEB, SEC, *etc.*) cause food poisoning. TSST-1 and SEs are known as superantigens. *S. aureus* also produces a number of cytotoxic molecules that include four hemolysins (α- [Hla], β- [Hlb], δ- [Hld], and γ- [Hlg] toxins). Production of these virulence factors in *S. aureus* is carefully controlled in response to cell density (quorum sensing), energy availability, and environmental signals by accessory gene regulators including Agr, Sar, Sae, and others [10]. These global regulators also control surface proteins (adhesins), such as 2 fibronectin-binding proteins A and B (FnBPA and FnBPB), 2 fibrinogen-binding proteins known as clumping factors A and B (ClfA and ClfB), and a collagen-binding protein (Cna), which are responsible for the adherence, colonization, and biofilm formation of MRSA isolates [5, 7, 10, 11]. However, no clear mechanism has been elucidated for biofilm formation and pathogenicity of *S. aureus* infections of the urinary tract.

In the present study, we investigated the relationship between biofilm-forming capacities and virulence determinants/clinical background of 109 MRSA isolates collected from patients with UTI over a 12-year period from 1990 to 2001 at the Department of Urology, Okayama University Hospital. We analyzed the presence of genes encoding superantigens (*tst, sea, seb, sec*), hemolysins (*hla, hlb*), surface proteins (*fnbA, fnbB, clfA, cna*), PIA (*icaA*), and global regulators (*agrI, agrII, agrIII, and agrIV* subgroup) in the MRSA isolates and retrospectively reviewed the associated medical records.

Materials and Methods

Bacterial isolates. The bacterial isolates used in this study were MRSA isolated from patients with UTI at the Department of Urology, Okayama University Hospital, over a 12-year period from 1990 through 2001. A total of 109 isolates that grew to $> 10^4$ CFU/ml in urinary culture were selected for this study. All 109 patients (one isolate per patient) had documented pyuria (WBC > 5/hpf). MRSA was defined as an *S. aureus* isolate possessing the *mecA* gene [4].

Biofilm formation assay. MRSA isolates were grown overnight at 37 °C in brain heart infusion broth supplemented with 2% glucose and 2% sucrose [12]. The culture was diluted 1:100 in medium, and 150 μl of this cell suspension was used to inoculate sterile flat-bottomed 96-well polystyrene microtiter plates (Corning Inc., Corning, NY, USA). After 48 h at 37 °C without shaking, wells were gently washed three times with 300 μl of distilled water, dried in an inverted position, and stained with 300 μl of 2% crystal violet solution in water for 45 min. After staining, plates were washed 3 times with distilled water. Quantitative analysis of biofilm production was performed by adding 200 μl of ethanol-acetic acid (95:5, vol/vol) to destain the wells. One hundred microliters from each well was transferred to a new microtiter plate, and the level (optical density; OD) of crystal violet present in the destaining solution was measured at 570 nm using a microtiter plate reader (Seikagaku Co., Tokyo, Japan). Each assay was performed in triplicate. As a control, uninoculated medium was used to determine background OD. The mean OD_{570} value from the control wells was subtracted from the mean OD_{570} value of tested wells.

Polymerase chain reaction (PCR) assay. PCR assays were performed to detect various genes in the MRSA isolates. The primers and PCR conditions used in this study are summarized in Table 1. Total cellular DNA was prepared as follows: 0.5 ml of MRSA culture, grown overnight in brain heart infusion broth (Nissui, Tokyo, Japan), was centrifuged, and the pellet was resuspended in 50 μl of InstaGene (Bio-Rad Laboratories, Hercules, CA, USA). After the suspension was heated for 10 min at 100 °C, 2.5 μl (or 5 μl for detection of *agrI, agrII, agrIII,* and *agrIV*) of the supernatant was mixed with 22.5 μl (or 20 μl for detection of *agrI, agrII, agrIII, and agrIV*) of premade reaction mixture to start the reaction. The primer pairs (2.5 pmol) for *tst, sea, seb, sec, hla, hlb, fnbA, fnbB, clfA, cna,* and *icaA*, and those (5 pmol) for *agrI, agrII, agrIII*, and *agrIV* were added to the respective reaction mixtures. The 25-μl reaction volume contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, $MgCl_2$ (concentrations shown in Table 1), 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 0.625 U of

Taq DNA polymerase (Takara Shuzo, Shiga, Japan). DNA amplification was carried out using the following thermal cycling profile: initial denaturation at 94 °C for 5 min, the denaturation, annealing, and extension reactions shown in Table 1 for 30 cycles (35 cycles for detection of *agrI, agrII, agrIII,* and *agrIV*), respectively, followed by final extension at 72 °C for 7 min. PCR products were then analyzed by electrophoresis on a 2% agarose gel. After electrophoresis, gels were stained with ethidium bromide (1 mg/l) and photographed under a UV transilluminator. A 100-bp DNA Ladder (New England Biolabs, Beverly, MA, USA) was used as a molecular size marker. The fragment sizes of each PCR product are shown in Table 1.

Retrospective clinical study. We retrospectively reviewed the medical records of the 109 patients and classified their UTI as catheter-related or catheter-unrelated, polymicrobial or monomicrobial, and febrile or non-febrile cases. Febrile UTI was defined as UTI in a patient with a body temperature of $\geq$ 38.0 °C.

Statistical methods. Data are expressed as mean values ± standard deviation (SD). Comparison of OD_{570} values between groups was carried out using Fisher's exact test or Mann-Whitney's *U* test. All results were considered statistically significant at the $P < 0.05$ level.

Results

Biofilm formation. Of the 109 MRSA isolates, 10 (9.2%), 31 (28.4%), 63 (57.8%), and 5 (4.6%) isolates exhibited strong (OD_{570} $\geq$0.5), medium (OD_{570} $\geq$0.2 to < 0.5), weak (OD_{570} 0 to < 0.2), and no biofilm formation, respectively. The mean OD_{570} of the 109

Table 1 PCR primers and conditions used in this study

Primer specificity	Primer sequences	Product length [bp]	PCR conditions cycling	$MgCl_2$ (mM)	Reference
tst	F: 5'-ATGGCAGCATCAGCTTGATA-3' R: 5'-TTTCCAATAACCACCCGTTT-3'	350	1 min, 94 °C; 1 min, 55 °C; 1 min, 72 °C	1.5	13
sea	F: 5'-TTGGAAACGGTTAAAACGAA-3' R: 5'-GAACCTTCCCATCAAAAACA-3'	120	1 min, 94 °C; 1 min, 55 °C; 1 min, 72 °C	1.5	13
seb	F: 5'-TCGCATCAAACTGACAAACG-3' R: 5'-GCAGGTACTCTATAAGTGCC-3'	478	1 min, 94 °C; 1 min, 55 °C; 1 min, 72 °C	1.5	13
sec	F: 5'-GACATAAAAGCTAGGAATTT-3' R: 5'-AAATCGGATTAACATTATCC-3'	257	1 min, 94 °C; 1 min, 55 °C; 1 min, 72 °C	1.5	13
hla	F: 5'-CTGGCCTTCAGCCTTTAAGG-3' R: 5'-CTGTAGCGAAGTCTGGTGAAA-3'	455	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	1.5	this study
hlb	F: 5'-GCCAAAGCCGAATCTAAG-3' R: 5'-CGCATATACATCCCATGGC-3'	845	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	1.5	14
fnbA	F: 5'-GCGGAGATCAAAGACAA-3' R: 5'-CCATCTATAGCTGTGTGG-3'	1278	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	1.5	14
fnbB	F: 5'-GGAGAAGGAATTAAGGCG-3' R: 5'-GCCGTCGCCTTGAGCGT-3'	811	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	1.5	14
clfA	F: 5'-CGATTGGCGTGGCTTCAG-3' R: 5'-GCCAGTAGCCAATGTCAC-3'	1004	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	1.5	14
cna	F: 5'-AGGATCAGATTCAAGGTGGACAGCA-3' R: 5'-GAGTGCCTTCCCAAACCTTTTGAG-3'	711	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	1.5	this study
icaA	F: 5'-GATTATGTAATGTGCTTGGA-3' R: 5'-ACTACTGCTGCGTTAATAAT-3'	770	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	4	15
agr subgroup I	F: 5'-ATCGCAGCTTATAGTACTTGT-3' R: 5'-CTTGATTACGTTTATATTTCATC-3'	739	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	3	15
agr subgroup II	F: 5'-AACGCTTGCAGCAGTTTATTT-3' R: 5'-CGACATTATAAGTATTACAACA-3'	691	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	3	15
agr subgroup III	F: 5'-TATATAAATTGTGATTTTTTATTG-3' R: 5'-TTCTTTAAGAGTAAATTGAGAA-3'	712	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	3	15
agr subgroup IV	F: 5'-GTTGCTTCTTATAGTACATGTT-3' R: 5'-CTTAAAAATATAGTGATTCCAATA-3'	683	1 min, 94 °C; 1 min, 50 °C; 1 min, 72 °C	3	15

isolates was 0.24 ± 0.18 (mean ± SD).

Presence of various genes in MRSA isolates. Of the 109 MRSA isolates, 79 (72.5%), 8 (7.3%), 21 (19.3%), 78 (71.6%), 89 (81.7%), 73 (67.0 %), 79 (72.5%), 3 (2.8%), 84 (77.1%), 6 (5.5%), 108 (99.1%), 1 (0.9%), 99 (90.8%), 2 (1.8%), and 0 (0%) isolates possessed *tst, sea, seb, sec, hla, hlb, fnbA, fnbB, clfA, cna, icaA, agrI, agrII, agrIII,* and *agrIV*, respectively (Fig. 1). Eight determinants (*tst, sec, hla, hlb, fnbA, clfA, icaA,* and *agrII*) were found to be predominant among these isolates.

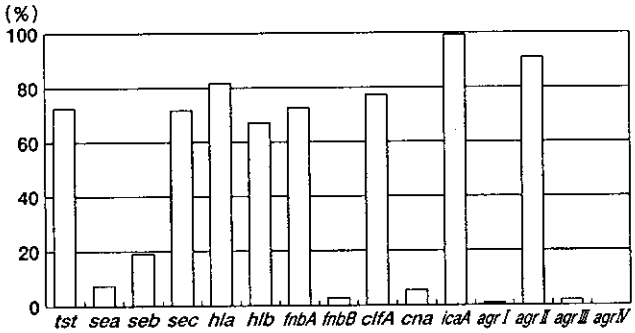

Fig. 1 Percentage of *tst*-, *sea*-, *seb*-, *sec*-, *hla*-, *hlb*-, *fnbA*-, *fnbB*-, *clfA*-, *cna*-, *icaA*-, *agrI*-, *agrII*-, *agrIII*-, and *agrIV*-positive isolates among MRSA isolates.

Relationship between biofilm formation and several virulence determinants. We evaluated the relationship between biofilm formation and the 6 predominant genes of the MRSA isolates (Table 2). The 6 determinants were as follows: *tst*, encoding the toxic shock syndrome toxin 1; *sec*, encoding the staphylococcal enterotoxin C; *hla*, encoding the α- toxin; *hlb*, encoding the β- toxin; *fnbA*, encoding the fibronectin-binding protein A; and *clfA*, encoding the fibrinogen-binding protein A. The other predominant genes, *icaA* of the intercellular adhesin locus and *agrII* of the accessory gene regulator, were excluded from the evaluation, since more than 90% of the MRSA isolates possessed these genes (Fig. 1). As shown in Table 2, the mean OD_{570} value (mean ± SD) was significantly higher in *hlb*- and *fnbA*-positive isolates than in *hlb*- and *fnbA*-negative isolates ($P = 0.0034$ and $P = 0.0052$, respectively). The value was also higher in *hla*-positive isolates than *hla*-negative isolates ($P = 0.0836$). The percentage of *hla*-, *hlb*-, and *fnbA*-positive isolates was

Table 2 Relationship between biofilm-forming capacities and several virulence determinants/clinical background

	No. of isolates	OD_{570} (mean ± SD)	*P* value (Mann-Whitney's *U* test)
Total isolates tested	109	0.24 ± 0.18	
Virulence determinants			
tst-positive	79	0.21 ± 0.29	0.7783
tst-negative	30	0.30 ± 0.46	
sec-positive	78	0.21 ± 0.29	0.6503
sec-negative	31	0.29 ± 0.45	
hla-positive	89	0.26 ± 0.38	0.0836
hla-negative	20	0.12 ± 0.08	
hlb-positive	73	0.29 ± 0.40	0.0034
hlb-negative	36	0.12 ± 0.11	
fnbA-positive	79	0.28 ± 0.39	0.0052
fnbA-negative	30	0.12 ± 0.12	
clfA-positive	84	0.23 ± 0.32	0.8996
clfA-negative	25	0.25 ± 0.43	
Clinical background			
catheter-related	51	0.29 ± 0.39	0.0162
catheter-unrelated	58	0.19 ± 0.30	
polymicrobial	50	0.25 ± 0.40	no significance
monomicrobial	59	0.22 ± 0.30	
febrile	21	0.18 ± 0.15	no significance
non-febrile	88	0.25 ± 0.38	

45.6%, 57.4%, and 50% among those with OD_{570} values of 0 to < 0.2, 90.3%, 80.7%, and 83.9% among those with OD_{570} values of ≥0.2 to < 0.5, and 100%, 90.3%, and 90% among those with OD_{570} values of ≥0.5, respectively (Fig. 2). The percentage of *hla*- and *fnbA*-positive isolates was significantly higher in the strong biofilm-forming group than in the weak group ($P = 0.012$, $P = 0.020$), and the percentage of *hlb*-positive isolates was higher in the strong biofilm-forming group than in the weak group ($P = 0.079$). We also evaluated the biofilm-forming capacities of MRSA isolates in seven groups based on the presence/absence of *hla*, *hlb*, and *fnbA* genes (Fig. 3). As shown with a box and whisker plot, the MRSA isolates with 3 or 2 combinations of *hla*, *hlb*, and *fnbA* genes had greater capacities for biofilm formation than did those lacking these three genes. The Mann-Whitney's *U* test of 2 mean OD_{570} values (mean ± SD), 0.31 ± 0.43 and 0.15 ± 0.01, in 62 *hla*-, *hlb*-, *fnbA*-positive and 13 *hla*-, *hlb*-, *fnbA*-negative isolates, respectively, confirmed that MRSA isolates possessing *hla*, *hlb*, and *fnbA* genes together had significantly greater capacities for biofilm formation than did those lacking these 3 genes ($P = 0.0186$).

Relationship between biofilm formation and clinical background. The 109 cases of UTI caused by MRSA consisted of 51 catheter-related (46.8%) and 58 catheter-unrelated cases (53.2%), 50 polymicrobial (45.9%) and 59 monomicrobial cases (54.1%), and 21 febrile (19.3%) and 88 non-febrile cases (80.7%). The biofilm-forming capacities of MRSA isolates from catheter-related cases were significantly greater than those from catheter-unrelated cases ($P = 0.0162$) (Table 2). As shown in Fig. 4, the percentage of *hla*-, *hlb*-, and *fnbA*-positive isolates was 88.2%, 72.5%, and 76.5%, respectively, among MRSA isolates from catheter-related cases (n = 51) and 75.9%, 60.3%, and 70.7%, respectively, among those from catheter-unrelated cases (n = 58).

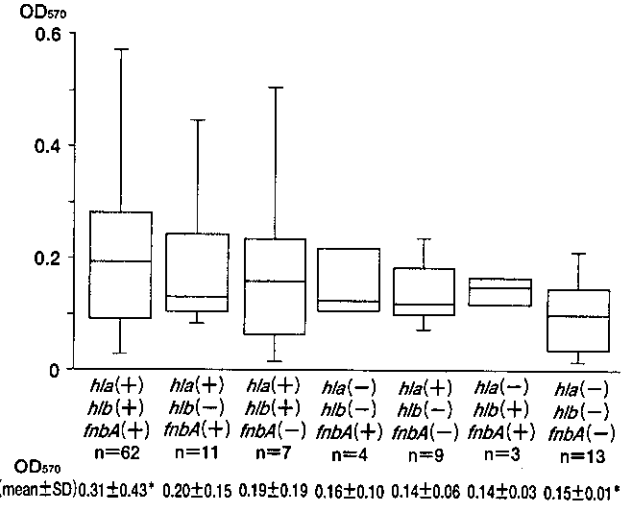

Fig. 3 Biofilm-forming capacities of MRSA isolates in seven groups based on the presence/absence of *hla*, *hlb*, and *fnbA* genes. OD_{570} values of the isolates in seven groups are shown by the box and whiskers plot that is a five-number summary (upper extreme, upper quartile, median, lower quartile, and lower extreme). The mean OD_{570} values (mean ± SD) in seven groups are also shown. *P = 0.0186 (Mann-Whitney's *U* test)

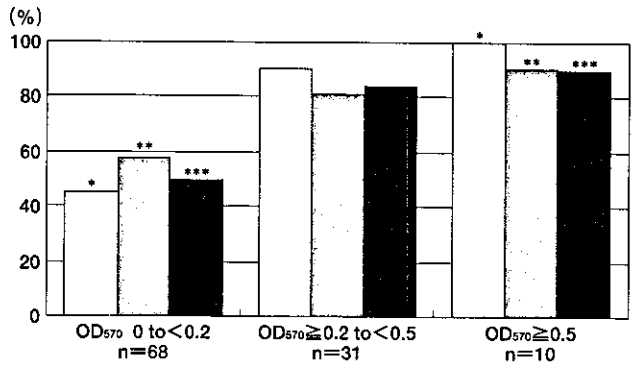

Fig. 2 Percentage of *hla*-, *hlb*-, *and fnbA*-positive isolates among MRSA isolates that belong to the following three biofilm-forming groups: OD_{570} 0 to < 0.2 (weak biofilm former), OD_{570} ≥ 0.2 to < 0.5 (medium biofilm former), and OD_{570} ≥ 0.5 (strong biofilm former). Bars: □, *hla* ; ▩, *hlb* ; ■, *fnbA*. *P = 0.012, **P = 0.079, ***P = 0.020 (Fisher's exact test)

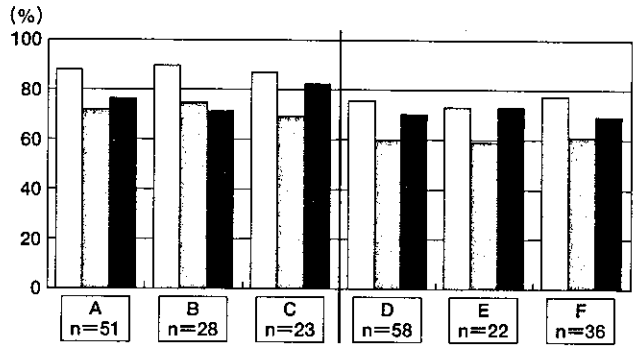

Fig. 4 Percentage of *hla*-, *hlb*-, and *fnbA*-positive isolates among MRSA isolates, from catheter-related cases (A), catheter-related polymicrobial cases (B), catheter-related monomicrobial cases (C), catheter-unrelated cases (D), catheter-unrelated polymicrobial cases (E), and catheter-unrelated monomicrobial cases (F). Bars: □, *hla* ; ▩, *hlb* ; ■, *fnbA*.

Discussion

On our urology ward, 20% of UTI caused by MRSA are febrile and patients who are asymptomatic are often observed without any intervention [4]. We previously reported that the presence of both the *tst* and *sec* genes may be associated with the incidence of febrile cases of UTI caused by MRSA [4]. MRSA isolated from urine rarely causes serious infectious symptoms, but once this occurs, therapy is difficult. One reason for this is that MRSA forms biofilms in the urinary tract [16]. It is also difficult to eradicate bacteria completely in patients with an indwelling urinary catheter and/or stent. Therefore, it is important to understand biofilm formation and the pathogenicity of MRSA infections in the urinary tract.

Biofilms are surface-associated, sessile bacterial communities. A mature biofilm is formed when planktonic cells initially colonize a surface, aggregate and/or grow into multicellular colonies, and embed themselves in an exopolysaccharide matrix [6, 7]. In general, staphylococcal cells embedded in a biofilm or in microcolonies are much more resistant to antimicrobial agents than are planktonic cells [5]. Many patients with a chronic staphylococcal infection have been treated with various antimicrobial agents, mostly without much success. Genetic analyses of staphylococci have shown that the progression of biofilm development consists of 2 steps: initial cell-to-surface interactions followed by cell-to-cell interactions [5, 11, 17].

S. aureus is especially capable of adhering to a large variety of matrix components to initiate colonization [5]. This adherence is frequently mediated by protein adhesins of the family known as MSCRAMM (microbial surface components recognizing adhesive matrix molecules). The collagen-binding protein, fibronectin-binding proteins, and fibrinogen-binding proteins belong to this family. In this study, we analyzed the presence of four determinants (*fnbA, fnbB, clfA,* and *cna*) encoding surface proteins FnBPA, FnBPB, ClfA, and Cna, respectively. Of 109 MRSA isolates, 79 (72.5%), 3 (2.8%), 84 (77.1%), and 6 (5.5%) isolates possessed *fnbA, fnbB, clfA,* and *cna,* respectively. Of the four determinants, *fnbA* and *clfA* genes were predominant in the isolates, and the *fnbA*-positive isolates had significantly greater capacities for biofilm formation than did the *fnbA*-negative isolates ($P = 0.0052$) (Table 2). The percentage of *fnbA*-positive isolates was higher among MRSA isolates from catheter-related cases than those from catheter-unrelated cases (Fig. 4).

The *ica* locus, which is required for the synthesis of the polysaccharide intracellular adhesin (PIA) of staphylococci, plays a role in cell-to-cell interactions during biofilm formation and is predominantly present in clinical isolates [5]. Our data showed that 108 of 109 (99.1%) of MRSA isolates possessed *icaA*. Only one isolate without the *icaA* gene possessed *tst, sec, hla, hlb, fnbA,* and *clfA,* and the biofilm-forming capacity of the *icaA*-negative isolate was weak (OD_{570} value: 0.02). Even though the isolate does not produce PIA, other surface-associated virulence factors may be overexpressed, functionally compensating for the lack of PIA. Other factors, such as the autolysin, the D-alanine esterification of teichoic acids, the accumulation-associated protein, and the like that contribute to biofilm formation were described in a review article [5]. Previously unknown factors, in particular adhesins, which have been identified by whole genome sequencing of MRSA, may also contribute to biofilm formation [18].

Caiazza *et al.* [8] showed that Hla, a 34-kDa protein that causes host cell lysis by heptamerizing upon insertion into eukaryotic cell membranes, plays a role primarily in cell-to-cell interactions during biofilm formation. The level of Hla correlates with the level of biofilm formation. Caiazza *et al.* [8] were initially surprised to find that a secreted toxin had such a dramatic impact on biofilm formation. In this study, we showed that the biofilm-forming capacities of MRSA isolates were higher in *hla*- and *hlb*-positive isolates than in *hla*- and *hlb*-negative isolates, respectively ($P = 0.0034$, $P = 0.0836$). Other examples exist in which secreted toxins and enzymes may play a role in biofilm formation [19, 20, 21, 22]. These toxins encoded by *hla* and *hlb* genes may be bifunctional enzymes and cause tissue damage of urinary epithelium. The percentage of *hla*- and *hlb*-positive isolates was higher among MRSA isolates from catheter-related cases than those from catheter-unrelated cases (Fig. 4).

Quorum sensing via the accessory gene regulator (*agr*) system has been assigned a central role in the pathogenesis of staphylococci, particularly *S. aureus* [10, 11]. The *agr* system regulates a wide array of virulence factors, including those involved in surface-associated virulence and biofilm formation [5, 7, 10, 11]. *S. aureus* strains can be divided into 4 major groups based on *agr* variations [10]. The relationship between *agr* groups and clinical features has been reported [10, 23]; for instance, most menstrual TSS strains

belong to *agr* group III [24], all the strains causing leucocidin-induced necrotizing pneumonia belong to *agr* group III [25], most intermediate-level glycopeptide resistance strains belong to *agr* group II [26], and most exfoliatin-producing strains belong to *agr* group IV [27]. Our data showed that 99 of 109 (90.8%) of the MRSA isolates belonged to *agr* group II. We are unable to assess the relationship between *agr* group II and UTI, since *agr* group II may be predominant in MRSA isolated in Japan, based on a database search [18].

The epidemic of UTI caused by MRSA at the Okayama University Hospital appears to be representative of the changing epidemiology of *S. aureus* throughout Japan [4]. Molecular typing of MRSA isolates by random amplified polymorphic DNA and pulsed-field gel electrophoresis analyses revealed no apparent clonality of these isolates in the urology ward over a 10-year period. It is possible that the MRSA in the urology ward originate from other wards in the hospital, other hospitals, or other communities. MRSA isolates that had previously been largely confined to hospitals have recently started emerging in the outside community [28, 29, 30].

In this study, the biofilm-forming capacities of MRSA isolates from catheter-related cases were significantly greater than those from catheter-unrelated cases ($P = 0.0162$). Biofilm formation by staphylococci occurs not only with indwelling devices but also in response to any bacterial factor that mediates adherence to components of the extracellular matrix of the host [5]. Peacock *et al.* [15] reported that seven determinants (*fnbA, cna, sdrE, sej, eta, hlg*, and *ica*) of *S. aureus* were significantly more common in invasive isolates. No single factor predominated as the major predictor of virulence, and their effects appeared to be cumulative. The relative importance of host factors versus bacterial virulence determinants in disease pathogenesis is unknown. Host factors for *S. aureus* disease are likely to include a genetic predisposition via one or more susceptibility genes and acquired factors such as the presence of intravenous devices, surgical wounds, and other events that perturb normal host defenses.

Taken together, our studies suggest that MRSA colonization and infection of the urinary tract may be promoted by *hla, hlb*, and *fnbA* gene products.

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Clinical Implications of Biofilm Formation by *Enterococcus faecalis* in the Urinary Tract

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The potential relationships between biofilm formation and pathogenicity of *Enterococcus faecalis* in urinary tract infections (UTI) were investigated. Over a 12-year period from 1991 through 2002, a total of 352 *E. faecalis* isolates were collected from patients with complicated UTI (one isolate per patient) at the urology ward of Okayama University Hospital. We analyzed the prevalence and transferability of genes encoding virulence factors (*asa1*, *esp*, *cylA*, *gelE/sprE*) and antimicrobial resistance (*aac(6')/aph(2")*). The production of biofilm, hemolysin and gelatinase by these isolates was also examined and the associated medical records of patients were retrospectively reviewed. Of 352 *E. faecalis* isolates, 315 possessed *asa1* and/or *esp* genes. Of the 63 hemolysin- and 167 gelatinase-producing isolates, 59 and 94 isolates, respectively, possessed both *asa1* and *esp* genes. *E. faecalis* isolates with both *asa1* and *esp* genes formed biofilms at significantly higher rates than those with neither gene (P=0.038). The genes encoding *asa1*, *cylA* and *aac(6')/aph(2")* were transferable and appeared to have accumulated in these isolates. The *E. faecalis* isolates possessing *asa1* and/or *esp* genes were found from both catheter-related or -unrelated UTI. Our study indicates that *E. faecalis* isolates that have accumulated virulence genes are apt to form persistent biofilms in the urinary tract.

Key words: *Enterococcus faecalis*, urinary tract infection, biofilm formation, pathogenicity, gene transfer

Enterococcus faecalis is a normal commensal in the human intestinal flora but can lead to nosocomial infections [1-5]. Although the pathogenicity of *E. faecalis* in the urinary tract is not considered high, *E. faecalis* has been isolated from the urinary tract at increasing frequencies: up to 20% of urinary isolates in some reports [6-8]. Several virulence factors have been described in *E. faecalis*, including aggregation substance (Agg), enterococcal surface protein (Esp), cytolysin (Cyl) having both hemolytic and bactericidal activity, and gelatinase (Gel) [1, 3, 4]. These factors have been thought to act synergistically to enhance virulence by facilitating achievement of a quorum and activating the quorum-sensing mode of regulation, resulting in tissue damage and potentially deeper tissue invasion [1, 9-12]. Recent studies have shown that enterococci form biofilms and that the *E. faecalis fsr* quorum-sensing system controls biofilm development [13-17].

Agg is a surface protein expressed by the *asa1* gene, which is located on pheromone-responsive *E. faecalis* plasmids [18, 19]. It is produced in response to pheromones secreted by potential recipient *E. faecalis* cells, and causes the aggregation of donor and recipient cells, thereby facilitating the transfer of plasmids that may carry virulence traits and antibiotic resistance genes [18, 19]. Agg may also play an important role in the onset of enterococcal infection by facilitating the adherence of enterococci to cardiac vegetations as well as to the epithelial cells of the intestine, kidney and urinary tract [1]. Esp encoded by the chromosomal *esp* is associated with increased virulence, colonization and persistence in the urinary tract, along with biofilm formation [1, 13, 17, 20, 21]. The production of Cyl has also been shown to significantly worsen the severity of endocarditis and endophthalmitis in animal models as well as to contribute to the severity of enterococcal disease in humans [1, 22]. Cyl

is either encoded within pheromone-responsive plasmids or on the chromosome within pathogenicity islands and is transcribed as an operon that contains at least five genes including *cylA* [1, 22]. Gel, encoded by the chromosomal *gelE*, is an extracellular zinc-metalloprotease that hydrolyzes collagen, gelatin, and small peptides and that has been shown to exacerbate endocarditis in an animal model [1].

In the present study, we investigated the potential relationships between biofilm formation and clinical implications of *E. faecalis* isolates in the urinary tract. Over a 12-year period from 1991 through 2002, a total of 352 *E. faecalis* isolates were collected from patients with complicated urinary tract infection (UTI) at the urology ward of Okayama University Hospital. We analyzed the prevalence and transferability of genes encoding virulence factors (*asa1*, *esp*, *cylA*, *gelE/sprE*) and antimicrobial resistance (*aac(6')/aph(2")*). In addition, the production by these isolates of biofilm and extracellular enzymes, hemolysin (Hln) and gelatinase was examined, and the associated medical records of the patients were retrospectively reviewed. The data were summarized in four groups based on the presence/absence of *asa1* and *esp* genes encoding enterococcal adhesins, Agg and Esp, respectively.

Materials and Methods

Bacterial isolates from patients with UTI. The *E. faecalis* bacterial isolates used in this study were isolated from patients with complicated UTI at the Department of Urology, Okayama University Hospital, over a 12-year period from 1991 through 2002. A total of 352 isolates that grew to $\geq 10^4$ CFU/ml in urinary culture were selected for this study. All 352 patients (one isolate per patient) had documented pyuria (WBC ≥5/hpf).

Polymerase chain reaction (PCR) assay. The presence of *asa1*, *esp*, *cylA* *gelE/sprE* and *aac(6')/aph(2")*, which encode Agg, Esp, Cyl, Gel/serine protease and gentamicin resistance, respectively, was confirmed by PCR assay. The primers and PCR conditions used in this study are summarized in Table 1. Multiplex PCR assay was used to detect the *asa1* and *cylA* genes using primers reported by Huycke *et al.* [23]. Primers reported by Shankar *et al.* [20] were used for amplification within the N-terminal region of *esp*. Primers reported by Nakayama *et al.* [24] and Van de Klundert *et al.* [25] were used to amplify *gelE/sprE* and *aac(6')/aph(2")* genes, respectively. DNA amplification was carried out by the method of Kariyama *et al.* [26]. Briefly, total cellular DNA was prepared as follows: 0.5 ml of *E. faecalis* culture, grown overnight in Todd Hewitt broth (Becton Dickinson and Company, Sparks, MD, USA), was centrifuged, and the pellet was resuspended in 50 μl of InstaGene (Bio-Rad Laboratories, Hercules, CA, USA). After the suspension was heated for 10 min at 100°C, 2.5 μl of the supernatant was mixed with 22.5 μl of prepared reaction mixture to start the reaction. The primer pairs were added to the respective reaction mixtures. The 25-μl reaction volume contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 0.625 U of *Taq* DNA polymerase (Takara Shuzo, Shiga, Japan). PCR products were analyzed by electrophoresis on a 2% agarose gel. After electrophoresis, gels were stained with ethidium bromide (1 mg/l) and photographed under a UV trans-illuminator. A 100-bp DNA Ladder (New England Biolabs, Beverly, MA, USA) was used as a molecular size marker. The fragment sizes of PCR products are shown in Table 1.

Detection of hemolysin-producing isolate. Production of hemolysin was

determined by plating *E. faecalis* isolates onto Todd Hewitt agar plates supplemented with 5% rabbit blood and incubated at 37°C for 48 h. When hemolysis was observed on the plate at 48 h, the isolate was considered a hemolysin-producing isolate.

Detection of gelatinase-producing isolate. Production of gelatinase was determined by the method of Su *et al.* [27]. A transparent halo around colonies after exposure to a solution saturated with ammonium sulfate on the surface of the medium was considered a gelatinase-positive response.

Biofilm formation assay. *E. faecalis* isolates were grown overnight at 37°C in tryptic soy broth supplemented with 0.25% glucose [15]. The culture was diluted 1:100 in medium, and 200 μl of this cell suspension was used to inoculate sterile flat-bottomed 96-well polystyrene microtiter plates (Corning Inc., Corning, NY, USA). After 24 h at 37°C without shaking, wells were gently washed three times with 300 μl of distilled water, dried in an inverted position, and stained with 300 μl of 2% crystal violet solution in water for 45 min. After staining, plates were washed 3 times with distilled water. Quantitative analysis of biofilm production was performed by adding 300 μl of ethanol-acetic acid (95:5, vol/vol) to destain the wells. One hundred microliters from each well was transferred to a new microtiter plate, and the level (optical density; OD) of crystal violet present in the destaining solution was measured at 570 nm using a microtiter plate reader (Seikagaku Co., Tokyo, Japan). Each assay was performed in triplicate. As a control, uninoculated medium was used to determine background OD. The mean OD_{570} value from the control wells was subtracted from the mean OD_{570} value of tested wells.

Conjugative transfer experiments. Mating experiments were performed by the method of Clewell *et al.* [28]. Each of 43 *E. faecalis* isolates possessing the 3