JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2006, p. 2721–2727 Vol. 44, No. 8
0095-1137/06/$08.00+0 doi:10.1128/JCM.00512-06

Therapeutic Failures of Antibiotics Used To Treat Macrolide-Susceptible *Streptococcus pyogenes* Infections May Be Due to Biofilm Formation

Lucilla Baldassarri,[1]* Roberta Creti,[1] Simona Recchia,[1] Monica Imperi,[1] Bruna Facinelli,[2] Eleonora Giovanetti,[2] Marco Pataracchia,[1] Giovanna Alfarone,[1] and Graziella Orefici[1]

Dipartimento di Malattie Infettive, Parassitarie ed Immunomediate, Istituto Superiore di Sanità, Roma,[1] and Istituto di Microbiologia e Scienze Biomediche, Università Politecnica delle Marche, Ancona,[2] Italy

Received 9 March 2006/Returned for modification 24 April 2006/Accepted 5 June 2006

Streptococcus pyogenes **infections often fail to respond to antibiotic therapy, leading to persistent throat carriage and recurrent infections. Such failures cannot always be explained by the occurrence of antibiotic resistance determinants, and it has been suggested that *S. pyogenes* may enter epithelial cells to escape antibiotic treatment. We investigated 289 *S. pyogenes* strains isolated from different clinical sources to evaluate their ability to form biofilm as an alternative method to escape antibiotic treatment and host defenses. Up to 90% of *S. pyogenes* isolates, from both invasive and noninvasive infections, were able to form biofilm. Specific *emm* types, such as *emm*6, appeared to be more likely to produce biofilm, although variations within strains belonging to the same type might suggest biofilm formation to be a trait of individual strains rather than a general attribute of a serotype. Interestingly, erythromycin-susceptible isolates formed a significantly thicker biofilm than resistant isolates ($P < 0.05$). Among resistant strains, those carrying the *erm* class determinants formed a less organized biofilm than the *mef*(A)-positive strains. Also, *prtF1* appeared to be negatively associated with the ability to form biofilm ($P < 0.01$). Preliminary data on a selection of strains indicated that biofilm-forming isolates entered epithelial cells with significantly lower efficiency than biofilm-negative strains. We suggest that *prtF1*-negative macrolide-susceptible or *mef*(A)-carrying isolates, which are poorly equipped to enter cells, may use biofilm to escape antimicrobial treatments and survive within the host. In this view, biofilm formation by *S. pyogenes* could be responsible for unexplained treatment failures and recurrences due to susceptible microorganisms.**

Biofilm formation is recognized as an important virulence factor for both opportunistic pathogens (such as coagulase-negative staphylococci or enterococci) and "true" pathogens (such as *Staphylococcus aureus* or *Pseudomonas aeruginosa*) (32).

Streptococcus pyogenes (group A streptococcus [GAS]) is an important human pathogen that causes a variety of clinical manifestations ranging from noninvasive diseases, such as pharyngitis and impetigo, to more-severe, invasive infections, including necrotizing fasciitis, sepsis, and toxic shock-like syndrome (14). A large number of secreted or cell-attached virulence factors expressed by this microorganism have been investigated so far (5, 14). As far as biofilm is concerned, streptococcal species such as *Streptococcus gordonii* and *Streptococcus mutans* are well-known biofilm formers (17, 26), and recent observations suggesting that biofilm may also have a role in *S. pyogenes* infections have been reported. Hidalgo-Grass and colleagues (24) have observed that structured communities appear to be present in necrotizing fasciitis lesions, and Neely et al. (30) found similar characteristics in a model of *S. pyogenes* myositis in zebrafish. Akiyama et al. (1) reported that *S. pyogenes* from a murine model of impetigo was embedded in glycocalyx, a feature also observed with human lesions.

While still uniformly susceptible to penicillin, *S. pyogenes* strains may be resistant to macrolides, with resistance rates which vary considerably in different countries (11) and which are particularly elevated in Italy (13, 37). The extensive study of erythromycin-resistant *S. pyogenes* isolates in Italy confirmed the well-established phenotypic heterogeneity of these organisms, which results from the differentiation into the M phenotype (accounting for ca. 50% of Italian resistant isolates), the constitutive macrolide-lincosamide-streptogramin B resistance phenotype (15 to 20%), and the inducible macrolide-lincosamide-streptogramin B resistance phenotype (30 to 35%) (23, 37).

At any rate, several cases of recurrent infections (4), treatment failures of streptococcal pharyngitis (4, 22), and persistent throat carriage of *S. pyogenes* (34) which cannot be explained by antibiotic resistance are often observed (10). Facinelli et al. (18) provided a possible explanation of such phenomena by showing that erythromycin-resistant *S. pyogenes* invades epithelial cells, thus escaping antimicrobial treatment and the host immune response, by means of the *prtF1* gene, which encodes a protein involved in bacterial internalization into cells (25) and which is strongly associated with erythromycin resistance. A prominent feature shared by the *S. pyogenes* protein F1 and other high-affinity fibronectin-binding proteins is a structure containing tandem repeats 32 to 50 amino acids long, found adjacent to the conserved C-terminal cell attachment domain (19). In particular, protein F1 contains two fibronectin-binding domains, of which the one located towards the C terminus of the molecule, repeat domain type 2 (RD2), has been reported to contain a variable number of repeats, ranging from 1 to 6 (29, 31, 35).

As biofilm is known to provide organisms with an improved

* Corresponding author. Mailing address: Reparto di Malattie Batteriche Respiratorie e Sistemiche, Dipartimento di Malattie Infettive, Parassitarie ed Immunomediate, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Roma, Italy. Phone: 39.06.4990.2092. Fax: 39.06.4938.7112. E-mail: baldassa@iss.it.

TABLE 1. *emm* types and antibiotic resistance gene patterns of *S. pyogenes* strains examined in this study

emm type	Total no. of isolates	No. of strains with antibiotic resistance gene pattern			
		mef(A)	*erm*(B)	*erm*(A)	Other(s)
*emm*1	35	2			
*emm*2	11	6	1		3 [*mef*(A) *tet*(O)]
*emm*3	25				
*emm*4	31	14	2		12 [11 *mef*(A) *tet*(O) and 1 *erm*(B) *tet*(O)]
*emm*5	10		1	1	
*emm*6	18		3		
*emm*9	6	1			
*emm*11	5				1 [*tet*(M)]
*emm*12	19	8	3		1 [*erm*(B) *tet*(M)]
*emm*13	1		1		
*emm*14	1		1		
*emm*18	9		1		1 [*erm*(B) *tet*(M)]
*emm*22	15		8	2	4 [*erm*(B) *tet*(M)]
*emm*24	3				
*emm*28	8		2		
*emm*33	1				
*emm*44/61	12	2	2		
*emm*48	1				
*emm*58	2				1 [*tet*(M)]
*emm*62	2		1		1 [*tet*(M)]
*emm*70	1				1 [*tet*(M)]
*emm*75	8	3			
*emm*76	1				
*emm*77	18		4	7	3 [*erm*(A) *tet*(O)]
*emm*78	10				
*emm*79	1				
*emm*87	1				2 [1 *tet*(M) and 1 *erm*(A) *tet*(O)]
*emm*89	25		20		1 [*erm*(B) *mef*(A)]
*emm*102	1				
*emm*108	4				
*emm*118	4				
Total	289	36	50	10	31

antibiotic resistance, besides supporting colonization and persistence, we started to investigate the ability of *S. pyogenes* to form biofilm in a plate test model (9) and found that several strains appeared to possess this characteristic (unpublished observation). Such evaluation was thus extended to a larger collection of *S. pyogenes* strains isolated from carriers, pharyngitis, and invasive diseases to evaluate a possible relationship between biofilm formation ability, clinical source, and antibiotic resistance pattern.

MATERIALS AND METHODS

Bacteria. A total of 162 antibiotic-susceptible and 127 erythromycin- and/or tetracycline-resistant *S. pyogenes* strains, collected in Italy between 1994 and 2005, were examined in this study. They included 133 strains isolated during two national enhanced surveillances on severe GAS infections conducted in Italy from 1994 to 1996 (36, 38) and 2003 to 2004 (the latter within the Fifth Framework Strep-Euro project on invasive GAS infections), 120 strains isolated from throat swabs of patients without clinical symptoms (6, 12), and 36 previously characterized strains isolated from pharyngitis (18, 35). Specifically, these pharyngeal strains had been characterized for the presence of the *prtF1* gene, RD2 types, cell invasiveness, and erythromycin resistance phenotype/genotype, including MIC and detection of resistance genes *erm*(A) subclass *erm*(TR), *erm*(B), and *mef*(A).

All strains (Table 1) had been *emm* typed previously by molecular methods (13; unpublished data).

Isolates were maintained in glycerol at −80°C and subcultured twice on sheep blood agar before testing. Todd-Hewitt broth (THB) was used for routine culture.

Antibiotic resistance. Macrolide resistance genes *erm*(B), *erm*(A) subclass *erm*(TR), and *mef*(A) and tetracycline resistance genes *tet*(O) and *tet*(M) were investigated by PCR as previously described (13, 23) (Table 1).

Biofilm formation. To test for biofilm formation, we used a quantitative adherence assay previously described (2). Briefly, a 1:10 dilution of overnight cultures in THB was used to inoculate wells in a microtiter polystyrene plate containing THB. After growth for 18 h at 37°C in unmodified atmosphere, atmosphere containing 5% CO_2, or anaerobiosis, plates were gently washed three times with phosphate-buffered saline (PBS), the adherent bacterial film was fixed by air drying at 60°C for 1 h and then stained with Hucker's crystal violet, and excess stain was washed off with tap water. The optical density (OD) of the biofilm was measured at 570 nm (OD_{570}) with an automatic spectrophotometer (Novapath microplate reader; Bio-Rad Laboratories, Inc., CA). To compensate for possible differences in growth rates under the different incubation conditions and/or for strains with different characteristics, e.g., antibiotic resistance, the adherence index was adjusted as an estimate of the density of the biofilm which would be generated by a culture with an OD_{600} of 0.5. Calculation of the adherence index was done according to the following formula: adherence index = mean density of biofilm (OD_{570}) × 0.5/mean growth (OD_{600}).

Strains that gave OD readings below 0.061 (mean ± 3 standard deviations [SD] of the blank) were classified as non-biofilm formers.

PCR for *prtF1*. The *prtF1* gene and the number of RD2 repeats within the gene were detected by PCR using the DNA primer pairs reported by Neeman et al. (31), as previously described (35). The number of RD2 repeats of *prtF1* was determined on the basis of the amplicon size, taking into account that one repeat was 111 or 96 bp long (34). Marker XIV (Roche Molecular Biochemicals, Mannheim, Germany) was used as the DNA size marker.

Scanning electron microscopy. Bacteria were grown in THB in 24-well plates containing polystyrene coverslips. After overnight incubation at 37°C, coverslips were rinsed twice in PBS and once in 0.1 M cacodylate buffer and fixed as previously described (2) to preserve extracellular polysaccharide. Briefly, cells were first fixed for 20 min with 0.1 M cacodylate-buffered 2.5% glutaraldehyde containing 0.075% (wt/vol) ruthenium red (Merck, Darmstadt, Germany) and 75 mM lysine at room temperature. They were then fixed with the same solution but without lysine for 2 h and finally fixed with 1% OsO_4 plus ruthenium red for an additional hour. Samples were dehydrated through a graded series of ethanol, critical point dried and gold sputtered, and examined by a Cambridge SE360 scanning electron microscope.

Cell invasion assay. Hep-2 cells (human epithelioid carcinoma; purchased from ATCC, Manassas, VA) were cultivated in minimal essential medium supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 10% fetal calf serum.

The ability of bacteria to invade epithelial cells was assayed by an antibiotic protection assay as previously described (3). Briefly, cells were seeded in 24-well plates at a concentration of 50,000 cells/ml. Bacteria grown overnight in THB were washed and suspended in the cell medium to give a bacterium-to-cell ratio of 100:1. Epithelial cells were infected for 2 h at 37°C, washed, and further incubated for 3 h in medium supplemented with 1% fetal calf serum and gentamicin, 200 μg/ml. At the different time points, duplicate wells were washed and lysed with 0.1% Triton X-100 in PBS for 5 min. Lysates were diluted and plated on Todd-Hewitt agar plates to enumerate viable bacteria. Cell invasion efficiency was defined by calculating the percentage of initial inoculum recovered after killing of extracellular bacteria with antibiotics (low efficiency, <0.1%; high efficiency, >0.1%).

Statistics. Significance of association of biofilm formation with the source of isolation was determined by the Student *t* test. Differences of optical density of biofilm were analyzed for significance by the Wilcoxon test for related rankable scores or the Mann-Whitney test; variance was analyzed according to Bonferroni-Dunn. All evaluations were done with StatView 4.1 (Abacus Concept) software on a Macintosh computer.

RESULTS

Culture conditions for biofilm formation. In preliminary experiments, we evaluated the influence of temperature (37°C versus 25°C) on 50 randomly chosen strains. Contrary to what was reported by Cho and Caparon (8), who found the lower temperature to support biofilm formation in *S. pyogenes* strain

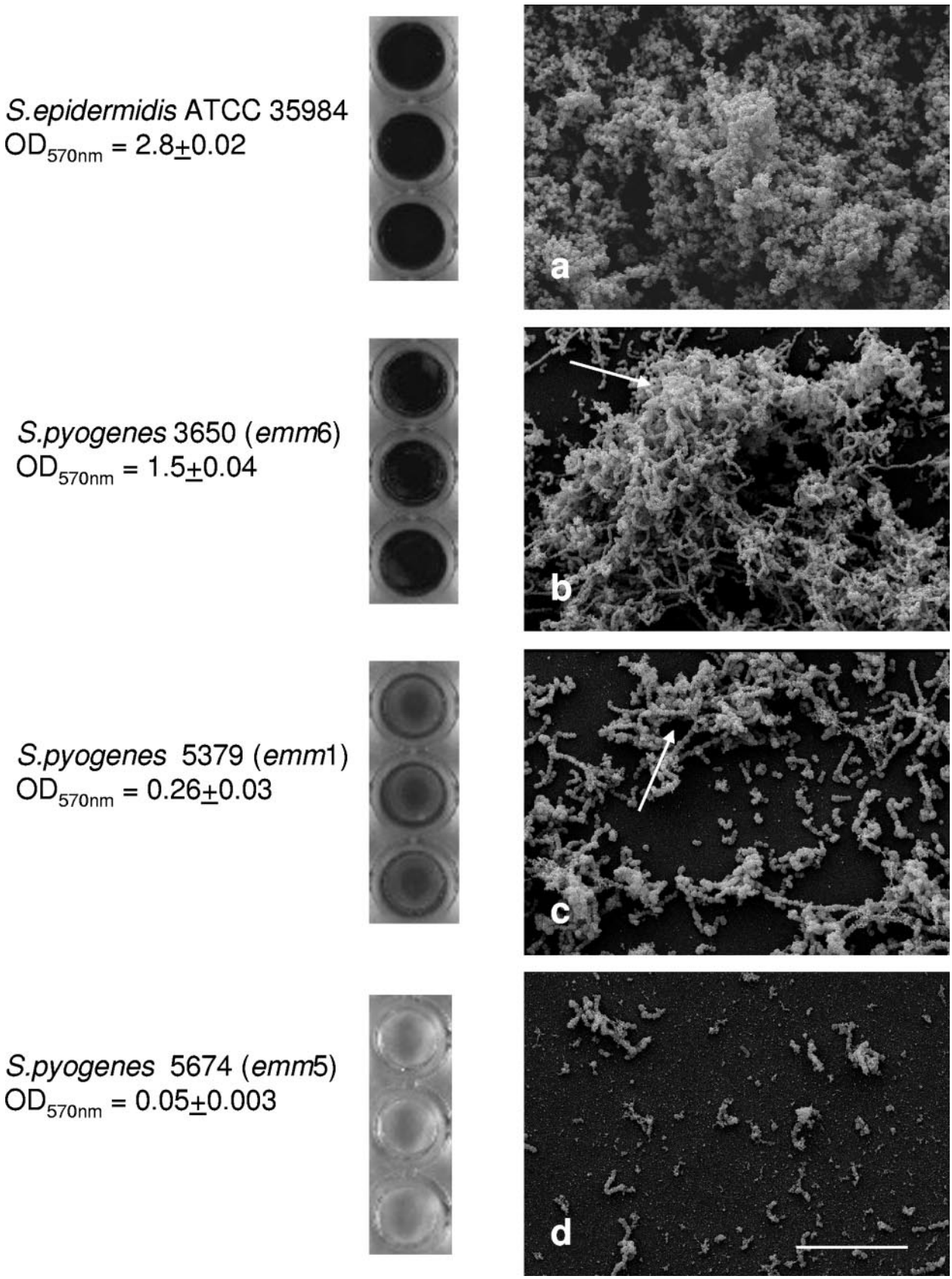

FIG. 1. Biofilms formed by *S. epidermidis* ATCC 35984 and three *S. pyogenes* strains with different biofilm-forming abilities. The ODs obtained with the plate test are compared with the biofilm appearances by scanning electron microscopy. The biofilms formed by *S. pyogenes* (b and c) appear to be less homogeneous than that of *S. epidermidis* (a), with large bacterial aggregates embedded in an amorphous extracellular matrix (arrows), coating the plastic surface more as scattered microcolonies. Bar, 20 μm.

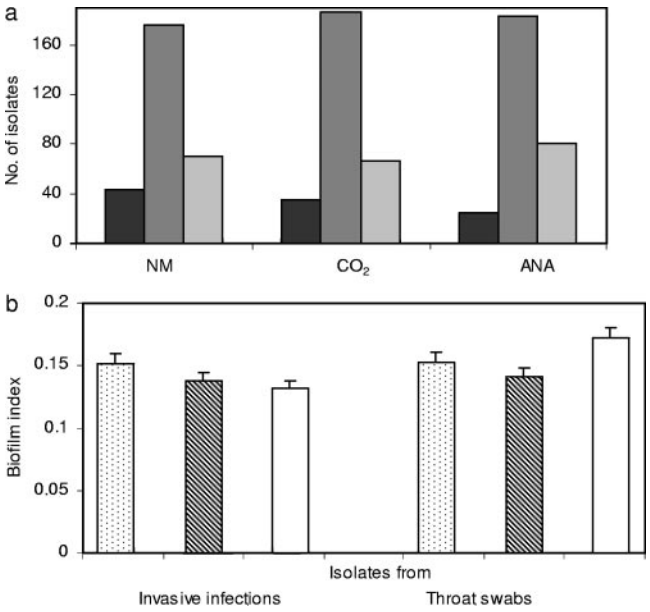

FIG. 2. (a) Numbers of *S. pyogenes* isolates forming biofilm at an OD of <0.061 (dark-gray bars), 0.061 < OD < 0.240 (gray bars), and an OD of >0.240 (light-gray bars) under the different atmosphere conditions. NM, unmodified atmosphere; ANA, anaerobiosis. (b) Index of biofilm produced in unmodified atmosphere (dotted bars), 5% CO_2 (striped bars), or anaerobiosis (open bars) by isolates from invasive infection or throat swabs. Bars indicate the mean ODs ± SD.

HSC5, we did not observe any difference (not shown). We thus decided to focus our attention more on conditions that *S. pyogenes* would encounter during infections, i.e., 37°C or higher temperatures, and different atmosphere compositions. Also, we determined that growth in THB without any specific supplement and incubation for 18 h appeared to be suitable conditions for biofilm formation. As a positive control, we used a known biofilm-forming microorganism, *Staphylococcus epidermidis* ATCC 35984 (9). To verify how the OD evaluation correlated with the biofilm architecture, three isolates with different biofilm forming abilities were also examined by scanning electron microscopy (Fig. 1).

Percentages of isolates that gave an OD reading above the established baseline (OD > 0.061) varied from 85% in unmodified atmosphere to 88% in 5% CO_2 to 91.4% in anaerobiosis. As shown in Fig. 2a, the numbers of isolates with 0.061 < OD < 0.24 were comparable in the three atmosphere compositions, while those with an OD of >0.240 were slightly more common when strains were grown in anaerobiosis. Single isolates did not appear to be considerably affected by atmosphere conditions, with the vast majority of strains forming comparable amounts of biofilm under the tested conditions. Only in a few cases were strains producing significantly more biofilm under one condition than under the others observed. However, when considered as a whole, ODs differed significantly when growth in CO_2 and growth in anaerobiosis were compared, with anaerobiosis significantly enhancing biofilm formation (Bonferroni-Dunn $P = 0.0143$). Differences between ODs after growth in unmodified atmosphere and growth in anaerobiosis bordered on statistical significance (Bonferroni-Dunn $P = 0.169$). Parallel measurements of biofilm formation were performed both as described in Materials and Methods and by applying the modification suggested by O'Toole and Kolter (33) to the original protocol described by Christensen et al. (9), i.e., solubilization of crystal violet by ethanol before reading. We could not see any difference between the two protocols (not shown).

Biofilm formation appeared to be related to the source of isolation when growth in anaerobiosis was considered (Fig. 2b); in this case, a significant increase in biofilm OD was observed for strains from throat swabs and noninvasive infections compared to isolates from invasive infections (Bonferroni-Dunn $P = 0.0152$). In this view, all of the data reported below refer to biofilm formation in anaerobiosis, unless statistically different results were obtained from the various atmosphere conditions.

As shown in Fig. 3, certain *emm* types were more likely to produce biofilm. Very strong biofilm producers (the majority showing ODs of >1.00) could be found exclusively among *emm*6 isolates; also, 3 *emm*77 strains out of 18 tested gave very high OD readings.

Macrolide resistance and biofilm formation. PCR analysis investigating macrolide resistance genes revealed 122 strains possessing *erm*(B) (50 strains), *mef*(A) (36 strains), *erm*(A)

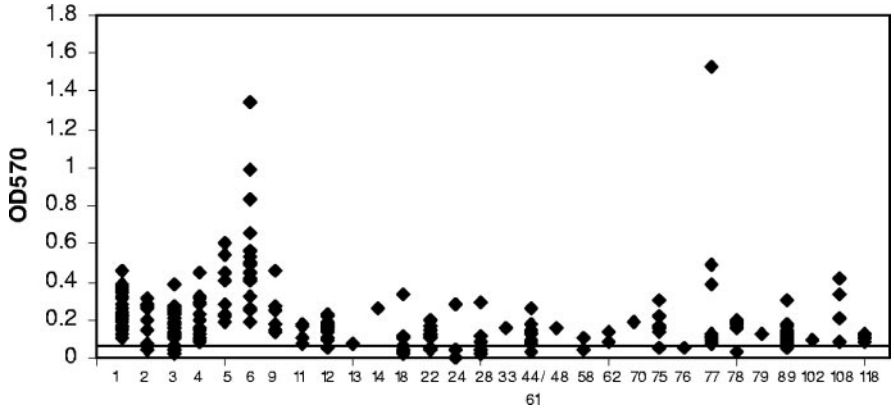

FIG. 3. Biofilm formation by *S. pyogenes* in relation to *emm* type. Symbols represent the means of at least three different determinations carried out in triplicate. Numbers along the *x* axis represent *emm* types (e.g., 1, *emm*1).

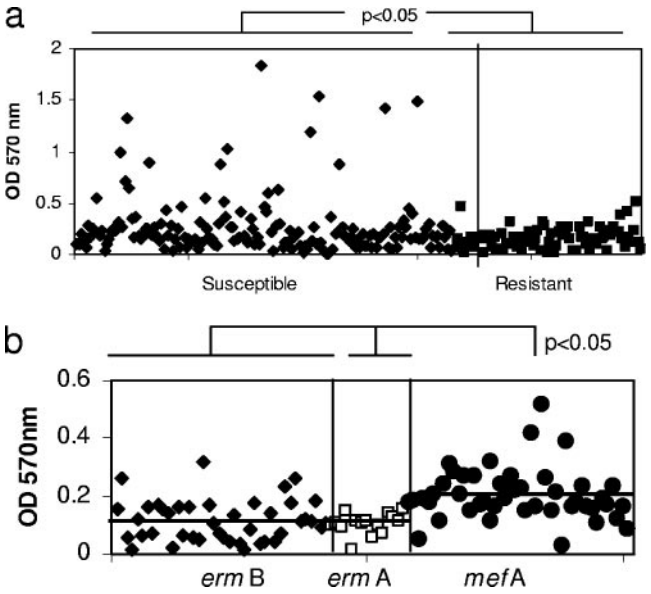

FIG. 4. Biofilm formation by antibiotic-susceptible and antibiotic-resistant *S. pyogenes* isolates. (a) Antibiotic-susceptible strains produced significantly more biofilm than resistant strains ($P < 0.05$). (b) Moreover, macrolide-resistant strains carrying *erm* genes produced a less thick biofilm than strains resistant to macrolides by the efflux pump *mef*(A). Values reported here are those obtained after incubation in anaerobiosis. Symbols represent the mean ODs of three different determinations carried out in triplicate for each strain.

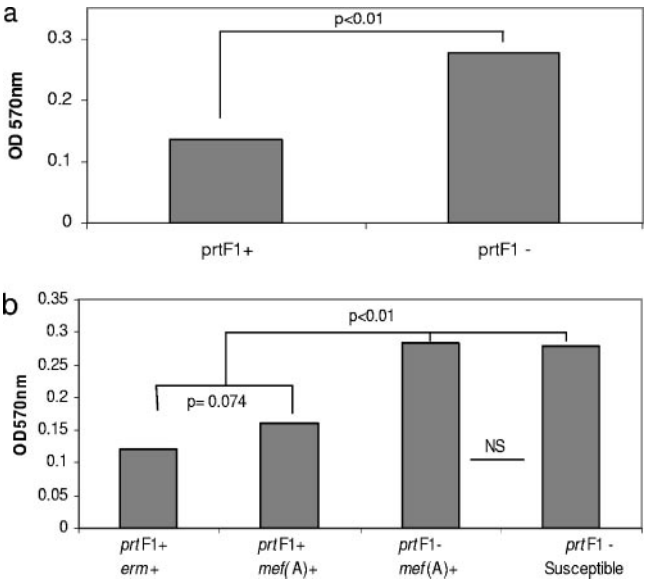

FIG. 5. Biofilm formation by *S. pyogenes* in relation to the presence or absence of the *prtF1* gene. (a) *prtF1*-negative strains formed a significantly thicker biofilm than *prtF1*-carrying strains ($P < 0.01$). (b) Biofilm formation in susceptible isolates or macrolide-resistant isolates containing either *erm* or *mef* and *prtF1* genes is shown. Bars are the median ODs of all isolates tested in triplicate at least three times. NS, not significant.

subclass *erm*(TR) (10 strains), or a combination of these (Table 1). Tetracycline resistance genes are specified in Table 1.

Macrolide-susceptible strains produced significantly more biofilm than resistant strains (mean and median ODs, 0.235 and 0.231 versus 0.141 and 0.133, respectively) (Fig. 4a). In particular, the presence of genes coding macrolide resistance mediated by methylation of 23S rRNA appeared to negatively affect the ability of strains to produce biofilm [mean and median ODs, 0.114 and 0.108 and 0.103 and 0.109 for *erm*(B)- and *erm*(A) subclass *erm*(TR)-positive strains, respectively] compared to results for susceptible strains and *mef*(A)-positive isolates (mean and median ODs, 0.202 and 0.185) (Fig. 4b). Tetracycline resistance did not appear to affect biofilm formation (not shown). Notably, the strongest biofilm producers, *emm*6 isolates, were susceptible to macrolides, as were the three strong biofilm-forming *emm*77 isolates, while all but one of the other *emm*77 isolates tested were *erm*(A) subclass *erm*(TR) or *erm*(B) positive.

Presence of *prtF1* gene and biofilm. As it was reported previously that erythromycin resistance was strongly related to the presence of the *prtF1* gene (18), we also examined this characteristic in a selection of our strains. Thirty-six strains had been characterized previously for the presence of the *prtF1* gene and for RD2 profile (35); the same characteristics were examined for 40 additional, randomly chosen, isolates. Overall, 76 strains were considered: 57 were positive for *prtF1*, and 19 did not give any PCR product with the primers used. As shown in Fig. 5a, a statistically significant increase in biofilm formation was observed between *prtF1*-negative and *prtF1*-positive isolates. When the number of RD2 repeats was investigated, we found 22 isolates with four repeats, 15 isolates with three repeats, only 3 isolates with two repeats, and 17 isolates with one repeat (not shown). The number of RD2 repeats inside the gene did not affect biofilm formation. The negative association between *prtF1* and macrolide resistance mediated by 23S rRNA methylation was also confirmed by grouping the isolates on the basis of antibiotic susceptibility, *erm* resistance, *mef* resistance, and presence/absence of *prtF1* (Fig. 5b).

Cell invasion efficiency versus biofilm formation. To verify a possible interference of biofilm with the ability of *S. pyogenes* to enter epithelial cells, seven biofilm-forming and seven biofilm-negative isolates were tested in an antibiotic protection assay with Hep-2 cells. Isolates that were able to invade cells with high efficiency were those producing the lowest amount of biofilm; the strong biofilm formers entered cells only with low efficiency (Fig. 6). Biofilm ODs of *S. pyogenes* isolates with high or low cell invasion efficiency differed significantly ($P < 0.01$).

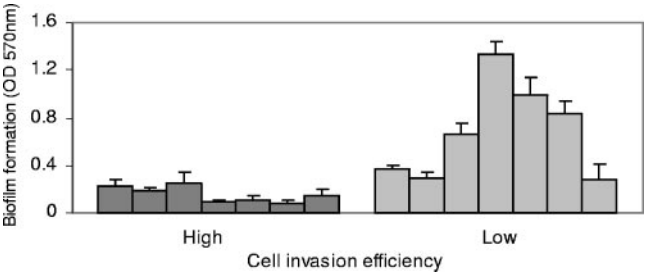

FIG. 6. Biofilm formation abilities of a selection of 14 *S. pyogenes* isolates in relation to efficiency in invading Hep-2 cells. Bars represent the mean ODs (±SD) of three different determinations carried out in triplicate for each strain. The difference in OD between the two groups was significant ($P < 0.01$).

DISCUSSION

Biofilm is known to provide microorganisms with greater resistance to antibiotic treatment and to host immunity (15). In this view, the ability to grow in protective mode would allow antibiotic-susceptible organisms a longer persistence in the host despite antimicrobial therapy.

In this first systematic analysis of a large collection of clinical isolates from different sources, we have shown that almost all *S. pyogenes* isolates are able to form biofilm, although to different extents, which may provide an additional possible explanation of treatment failures, recurrences, and/or persistence of streptococcal colonization. Anaerobiosis appeared to stimulate biofilm formation, particularly compared with growth in the presence of CO_2. Under the condition of anaerobiosis, biofilm formation appeared to be more common among throat and pharyngitis isolates than among strains from invasive infections. Different behaviors of pharyngitis isolates and isolates from other infection types with regard to the ability to enter epithelial cells have already been shown (28). It is reasonable that persistence of *S. pyogenes* on surfaces exposed to mechanical stress may be facilitated by biofilm formation. One possible explanation of the stimulating action of lower oxygen tensions on biofilm formation may reside in differential control by regulators at the transcriptional level, as already shown for other virulence traits of *S. pyogenes* (7, 20). Moreover, *S. pyogenes* may start to form biofilm during the early colonization stages even under nonoptimal conditions; as biofilm forms, the oxygen tension in the lower layers decreases, which may further stimulate biofilm growth.

The ability to form biofilm appeared to be related to the *emm* type, possibly due to the known linkage existing between *emm* types and other factors, such as phage-associated superantigen pattern or antibiotic resistance pattern (13, 16). This would be in accordance with the results reported by Cho and Caparon (8), who found M protein mutants to be unable to form biofilm, and further supported by the fact that expression of M protein, as with that of biofilm, has been shown to be stimulated in an O_2-poor environment (21). However, variations within strains belonging to the same *emm* type were also observed, suggesting biofilm formation to be a trait of individual strains rather than a general attribute of a serotype. A recent publication by Lembke et al. (27) also indicates a linkage between biofilm formation and *emm* type.

The most noteworthy information obtained from this study was the negative association of the ability to form biofilm with the genetic determinants of macrolide resistance. Of the *S. pyogenes* strains tested, those carrying the macrolide resistance *erm*(A) subclass *erm*(TR) and *erm*(B) methylase genes formed less-thick biofilm than susceptible isolates. On the other hand, neither the *mef*(A)-encoded macrolide efflux pump nor tetracycline resistance appeared to affect biofilm formation. The meaning of these findings may be that, in order to escape antibiotic treatment and support their survival, erythromycin-susceptible bacteria need alternative strategies which are of no use to resistant bacteria. Considering the biological cost, a hypothesis might be that the *erm*-mediated macrolide resistance is more expensive in terms of energy requirement than the *mef*(A)-mediated efflux resistance, although numerous other factors should be examined to confirm such speculation. It is worth noting that all biofilm data reported in this study were corrected to include possible differences in growth rates, thus excluding any effect due to less or slowed growth of resistant versus susceptible bacteria.

As previously suggested (18), *S. pyogenes* strains combining erythromycin resistance and the ability to enter human respiratory cells may be able to escape β-lactams by virtue of intracellular location and macrolides by virtue of resistance, resulting in difficulty of eradication and easier clonal spread. Along the same lines, biofilm may function as a barrier against antimicrobials, facilitating persistence of susceptible organisms and possibly supporting horizontal gene transfer and creation of virulent clones.

The same authors (18) have also shown that the proportion of *S. pyogenes* carrying *prtF1*, the internalization-associated gene, is significantly higher among erythromycin-resistant strains than among erythromycin-susceptible strains. Notably, the erythromycin-resistant strains showing a less consistent association with the *prtF1* gene and cellular invasion were those carrying the *mef*(A) gene, i.e., those forming more biofilm than *erm*-carrying isolates. These data indicate that macrolide-resistant, *prtF1*-carrying, cell-invading *S. pyogenes* isolates may not need biofilm to survive within the host. We have shown here results from a small subset of isolates where biofilm-producing strains invaded epithelial cells with lower efficiency than non-biofilm-forming strains. In support of both findings is the reported negative association of the *prtF1* gene with biofilm formation, where the gene-carrying strains formed less-thick biofilm.

It can thus reasonably be hypothesized that macrolide-susceptible *S. pyogenes* strains may use biofilm to escape antimicrobial treatments and survive within the host. As well, *mef*(A)-carrying strains, less able or poorly equipped to enter cells, might use biofilm to survive β-lactam treatment. Overall, these findings strongly support the role of biofilm in successful *S. pyogenes* colonization and in difficulty of eradication. Testing for biofilm formation by the plate test, affordable even to routine laboratories, might be paired with the antibiogram to predict possible cases of noneradication of the pathogen and/or to apply synergic treatments facilitating antibiotic passage through the biofilm layer. Further investigations to understand the role of biofilm in the survival strategy of *S. pyogenes* and the complex relationships between biofilm formation and *prtF1* or other genes encoding adhesion-supporting extracellular proteins are warranted.

ACKNOWLEDGMENTS

We thank P. E. Varaldo for critical review of the manuscript and helpful suggestions.

This work was partially supported by a European Union grant (StrepEuro, QLK2.CT.2002.01398) to G.O. This work was also partially supported by ISS-NIH grant no. 5303.

We do not have a commercial or other association that may pose a conflict of interest.

REFERENCES

1. **Akiyama, H., S. Morizane, O. Yamasaki, T. Oono, and K. Iwatsuki.** 2003. Assessment of *Streptococcus pyogenes* microcolony formation in infected skin by confocal laser scanning microscopy. J. Dermatol. Sci. **32:**193–199.
2. **Baldassarri, L., R. Cecchini, L. Bertuccini, M. G. Ammendolia, F. Iosi, C. R. Arciola, L. Montanaro, R. Di Rosa, G. Gherardi, G. Dicuonzo, G. Orefici, and R. Creti.** 2001. *Enterococcus* spp. produces slime and survives in rat peritoneal macrophages. Med. Microbiol. Immunol. **190:**113–120.

3. **Baldassarri, L., S. Recchia, M. Imperi, R. Creti, G. Alfarone, M. Pataracchia, and G. Orefici.** 2006. Fibronectin binding protein genes and cell invasion ability of *Streptococcus pyogenes* isolated from different sources. Int. Congr. Ser. **1289:** 243–245.
4. **Bisno, A. L., M. A. Gerber, J. M. Gwaltney, E. L. Kaplan, and R. H. Schwartz.** 2002. Diagnosis and management of group A streptococcal pharyngitis: practice guidelines for streptococcal pharyngitis. Clin. Infect. Dis. **35:**113–125.
5. **Bisno, A. L., M. O. Brito, and C. M. Collins.** 2003. Molecular basis of group A streptococcal virulence. Lancet Infect. Dis. **4:**191–200.
6. **Cardona, F., and G. Orefici.** 2001. Group A streptococcal infections and tic disorders in an Italian pediatric population. J. Pediatr. **138:**71–75.
7. **Chaussee, M. S., J. Liu, D. L. Stevens, and J. J. Ferretti.** 1997. Effects of environmental factors on streptococcal erythrogenic toxin A (speA) production by Streptococcus pyogenes. Adv. Exp. Med. Biol. **418:**551–554.
8. **Cho, K. H., and M. G. Caparon.** 2005. Patterns of virulence gene expression differ between biofilm and tissue communities of *Streptococcus pyogenes*. Mol. Microbiol. **57:**1545–1556.
9. **Christensen, G. D., W. A. Simpson, J. J. Younger, L. M. Baddour, F. F. Barrett, D. M. Melton, and E. H. Beachey.** 1985. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J. Clin. Microbiol. **22:**996–1006.
10. **Conley, J., M. E. Olson, L. S. Cook, H. Crei, V. Phan, and H. D. Davies.** 2003. Biofilm formation by group A streptococci: is there a relationship with treatment failure? J. Clin. Microbiol. **41:**4043–4048.
11. **Cornaglia, G., and A. Bryskier.** 2004. Macrolide resistance of *Streptococcus pyogenes*, p. 150–165. *In* J. C. Pechere and E. L. Kaplan (ed.), Streptococcal pharyngitis. Karger, Basel, Switzerland.
12. **Creti, R., F. Cardona, M. Pataracchia, C. von Hunolstein, G. Cundari, A. Romano, and G. Orefici.** 2004. Characterisation of group A streptococcal (GAS) isolates from children with tic disorders. Indian J. Med. Res. **119:** 174–178.
13. **Creti, R., G. Gherardi, M. Imperi, C. von Hunolstein, L. Baldassarri, M. Pataracchia, G. Alfarone, F. Cardona, G. Dicuonzo, and G. Orefici.** 2005. Association of group A streptococcal *emm* types with virulence traits and macrolide-resistance genes is independent of the source of isolation. J. Med. Microbiol. **54:**913–917.
14. **Cunningham, M. W.** 2000. Pathogenesis of group A streptococcal infections. Clin. Microbiol. Rev. **13:**470–511.
15. **Donlan, R. M., and J. W. Costerton.** 2002. Biofilm: survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. **15:**167–193.
16. **Ekelund, K., J. Darenberg, A. Norrby-Teglund, S. Hoffmann, D. Bang, P. Skinhoj, and H. B. Konradsen.** 2005. Variations in *emm* type among group A streptococcal isolates causing invasive and noninvasive infections in a nationwide study. J. Clin. Microbiol. **43:**3101–3109.
17. **Ellen, R. P., G. Lepine, and P. M. Nghiem.** 1997. In vitro models that support adhesion specificity in biofilms of oral bacteria. Adv. Dent. Res. **11:**33–42.
18. **Facinelli, B., C. Spinaci, G. Magi, E. Giovanetti, and P. E. Varaldo.** 2001. Association between erythromycin resistance and ability to enter human respiratory cells in group A streptococci. Lancet **358:**30–33.
19. **Fischetti, V. A.** 2000. Surface proteins on gram-positive bacteria, p. 11–24. *In* V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy, and J. I. Rood (ed.), Gram-positive pathogens. American Society for Microbiology, Washington, D.C.
20. **Fogg, G. C., and M. G. Caparon.** 1997. Constitutive expression of fibronectin binding in *Streptococcus pyogenes* as a result of anaerobic activation of *rofA*. J. Bacteriol. **179:**6172–6180.
21. **Gibson, C., G. Fogg, N. Okada, R. T. Geist, E. Hanski, and M. Caparon.** 1995. Regulation of host cell recognition in *Streptococcus pyogenes*. Dev. Biol. Stand. **85:**137–144.
22. **Gillespie, S. H.** 1998. Failure of penicillin in *Streptococcus pyogenes* pharyngeal infection. Lancet **352:**1954–1956.
23. **Giovanetti, E., A. Brenciani, R. Lupidi, M. C. Roberts, and P. E. Varaldo.** 2003. Presence of the *tet*(O) gene in erythromycin- and tetracycline-resistant strains in *Streptococcus pyogenes* and linkage with either the *mef*(A) or the *erm*(A) gene. Antimicrob. Agents Chemother. **47:**2844–2849.
24. **Hidalgo-Grass, C., M. Dan-Goor, A. Maly, Y. Eran, L. A. Kwinn, V. Nizet, M. Ravins, J. Jaffe, A. Peyser, A. E. Moses, and E. Hanski.** 2004. Effect of a bacterial pheromone peptide on host chemokine degradation in group A streptococcal necrotising soft-tissue infections. Lancet **363:**696–703.
25. **Jadoun, J., E. Ozeri, E. Burstein, E. Skutelsky, E. Hansky, and S. Sela.** 1998. Protein F1 is required for efficient entry of *Streptococcus pyogenes* into epithelial cells. J. Infect. Dis. **178:**147–158.
26. **Jefferson, K. K.** 2004. What drives bacteria to produce biofilm? FEMS Microbiol. Lett. **236:**163–173.
27. **Lembke, C., A. Podbielski, C. Hidalgo-Grass, L. Jonas, E. Hanski, and B. Kreikemeyer.** 2006. Characterization of biofilm formation by clinically relevant serotypes of group A streptococci. Appl. Environ. Microbiol. **72:**2864–2875.
28. **Molinari, G., and G. S. Chhatwal.** 1998. Invasion and survival of *Streptococcus pyogenes* in eukaryotic cells correlates with the source of clinical isolates. J. Infect. Dis. **177:**1600–1607.
29. **Natanson, S., S. Sela, A. E. Moses, J. M. Musser, M. G. Caparon, and E. Hanski.** 1995. Distribution of fibronectin-binding proteins among group A streptococci of different M types. J. Infect. Dis. **171:**871–878.
30. **Neely, M. N., J. D. Pfeifer, and M. Caparon.** 2002. Streptococcus-zebrafish model of bacterial pathogenesis. Infect. Immun. **70:**3904–3914.
31. **Neeman, R., N. Keller, A. Barzilai, Z. Korenman, and S. Sela.** 1998. Prevalence of internalization-associated gene, prtF1, among persisting group A streptococcus strains isolated from asymptomatic carriers. Lancet **352:**1974–1977.
32. **O'Toole, G., H. B. Kaplan, and R. Kolter.** 2000. Biofilm formation as microbial development. Annu. Rev. Microbiol. **40:**445–450.
33. **O'Toole, G. A., and R. Kolter.** 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple convergent signalling pathways: a genetic analysis. Mol. Microbiol. **28:**449–461.
34. **Sela, S., R. Neeman, N. Keller, and A. Barzilai.** 2000. Relationship between asymptomatic carriage of *Streptococcus pyogenes* and the ability of the strains to adhere and be internalised by cultured epithelial cells. J. Med. Microbiol. **49:**499–502.
35. **Spinaci, C., G. Magi, C. Zampaloni, L. A. Vitali, C. Paoletti, M. R. Catania, M. Prenna, L. Ferrante, S. Ripa, P. E. Varaldo, and B. Facinelli.** 2004. Genetic diversity of cell-invasive erythromycin-resistant and -susceptible group A streptococci determined by analysis of the *RD2* region of the *prtF1* gene. J. Clin. Microbiol. **42:**639–644.
36. **Suligoi, B., C. von Hunolstein, G. Orefici, F. Scopetti, M. Pataracchia, and D. Greco.** 1998. Surveillance of systemic invasive diseases caused by group A streptococcus in Italy 1994–1996. Euro Surveill. **3:**11–14.
37. **Varaldo, P. E., E. A. Debbia, G. Nicoletti, D. Pavesio, S. Ripa, G. C. Schito, G. Tempera, et al.** 1999. Nationwide survey in Italy of treatment of *Streptococcus pyogenes* pharyngitis in children: influence of macrolide resistance on clinical and microbiological outcomes. Clin. Infect. Dis. **29:**869–873.
38. **von Hunolstein, C., B. Suligoi, M. Pataracchia, F. Scopetti, S. Recchia, D. Greco, and G. Orefici.** 1997. Clinical and microbiological characteristics of severe group A streptococcal infections in Italy. Adv. Exp. Med. Biol. **418:** 79–81.