Journal of Applied Microbiology 2000, **88**, 546–553

Role of RpoS and AlgT in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide and monochloramine

W.L. Cochran[1,2], S.-J. Suh[4], G.A. McFeters[1,2] and P.S. Stewart[1,3]

[1]Center for Biofilm Engineering, [2]Department of Microbiology and [3]Department of Chemical Engineering, Montana State University, Bozeman, and [4]Department of Microbiology and Immunology, Medical College of Virginia, Campus of Virginia Commonwealth University, Richmond, USA

7326/07/99: received 30 July 1999, revised 15 November 1999 and accepted 22 November 1999

W.L. COCHRAN, S.-J. SUH, G.A. McFETERS AND P.S. STEWART. 2000. The role of two sigma factors, AlgT and RpoS, in mediating *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide and monochloramine was investigated. Two knock out mutant strains, SS24 (*rpoS*$^-$) and PAO6852 (*algT*$^-$), were compared with a wild type, PAO1, in their susceptibility to monochloramine and hydrogen peroxide. When grown as biofilms on alginate gel beads (mean untreated areal cell density $3{\cdot}7 \pm 0{\cdot}27$ log cfu cm^{-2}) or on glass slides (mean untreated areal cell density $7{\cdot}6 \pm 0{\cdot}9$ log cfu cm^{-2}), wild type bacteria exhibited reduced susceptibility to both antimicrobial agents in comparison with suspended cells. On alginate gel beads, all strains were equally resistant to monochloramine. *rpoS*$^-$ and *algT*$^-$ gel bead biofilms of 24-hour-old were more susceptible to hydrogen peroxide disinfection than were biofilms formed by PAO1. Biofilm disinfection rate coefficients for the two mutant strains were statistically indistinguishable from planktonic disinfection rate coefficients, indicating complete loss of biofilm resistance. While 48-hour-old *algT*$^-$ biofilm cells became resistant to hydrogen peroxide, 48-hour-old *rpoS*$^-$ biofilm cells remained highly susceptible. With the thicker biofilms formed on glass coupons, all strains were equally resistant to both hydrogen peroxide and monochloramine. It is concluded that while RpoS and AlgT may play a transient role in protecting thin biofilms from hydrogen peroxide, these sigma factors do not mediate resistance to monochloramine and do not contribute significantly to the hydrogen peroxide resistance of thick biofilms.

INTRODUCTION

Bacteria in biofilms exhibit reduced susceptibility, in comparison with freely suspended cells, to a wide variety of antimicrobial agents (Brown and Gilbert 1993). Although resistance mechanisms are important for controlling biofilms in diverse industrial and medical contexts, the mechanisms of biofilm resistance are not completely understood.

One hypothesized resistance mechanism is that the extracellular polymeric matrix of the biofilm constitutes a barrier to the inherent diffusive mobility of antimicrobial agents (Brown and Gilbert 1993). While solute diffusion in biofilms is somewhat retarded compared with diffusion in pure water, small solutes the size of disinfectants and antibiotics diffuse freely in biofilms (Stewart 1998). Given what is known about diffusion in biofilms, it is difficult to substantiate a generic physical barrier to antimicrobial diffusion (Stewart 1996). On the other hand, when the antimicrobial agent is reactively neutralized in the biofilm, slow or incomplete penetration can easily result (Stewart and Raquepas 1995; Stewart 1996). Bacteria or matrix constituents near the surface of the biofilm remove the biocide or antibiotic in large enough quantities to protect the bacteria more deeply embedded (Nichols *et al.* 1989; Brown and Gilbert 1993; Xu *et al.* 1996).

A second general hypothesis to explain biofilm resistance is that the cells in the biofilm are metabolically and physiologically different from planktonic cells (Nickel *et al.* 1985; Nichols *et al.* 1988; LeMagrex *et al.* 1994; Srinivasan *et al.* 1995; Sanderson and Stewart 1997; Cochran *et al.* 2000). An intriguing version of this hypothesis is that the biofilm phenotype is genetically programmed and results from the

Correspondence to: Dr P.S. Stewart, Department of Chemical Engineering, Montana State University, Bozeman, MT 59717, USA (e-mail: phil__s@erc.montana.edu).

up-regulation of banks of biofilm genes and the down-regulation of genes expressed primarily in planktonic culture. There are two regulatory genes that are particularly attractive candidates for involvement in mediating the biofilm physiology of *Pseudomonas aeruginosa*, *algT* and *rpoS*.

In the cystic fibrosis lung, mucoid strains of *Ps. aeruginosa* produce copious amounts of alginate. Alginate production is regulated by a cassette of genes, *algTmucABC* (Hershberger *et al*. 1995). AlgT is a sigma factor involved in the positive regulation of the mucoid phenotype in *Ps. aeruginosa*. MucA and MucB inhibit the production of AlgT in a negative feedback loop (Schurr *et al*. 1996; Xie *et al*. 1996). When spontaneous null mutations in *mucA* or *mucB* occur, AlgT is up-regulated, resulting in the increased production of alginate (Schurr *et al*. 1996; Xie *et al*. 1996). It has been suggested that alginate promotes biofilm formation (Boyd and Chakrabarty 1995) and it may be speculated that AlgT could function as a key factor in converting to the biofilm mode of growth. AlgT is also functionally interchangeable with RpoE, an extreme stress sigma factor found in the Enterbacteriaceae (Yu *et al*. 1995). Perhaps up-regulation of AlgT simultaneously induces biofilm formation and protection against extracellular stresses.

The alternative sigma factor, RpoS, has been identified as a central regulator of stationary phase-responsive genes (Lange and Hengge-Aronis 1991). In *Escherichia coli*, RpoS controls a large group of genes. Among the genes controlled are *katE* (Loewen *et al*. 1985), *katG* (Loewen *et al*. 1985; Ivanova *et al*. 1994) and *xthA* (Demple *et al*. 1983). All of these genes are involved in the protection of the cell against hydrogen peroxide. RpoS may also play a significant role in biofilm development. Structural and physiological heterogeneity of biofilms is now widely recognized (de Beer *et al*. 1994; Huang *et al*. 1995; Wentland *et al*. 1996; Huang *et al*. 1998), with regions of slow growth or reduced metabolic activity existing in close proximity to actively growing cells (Wentland *et al*. 1996; Huang *et al*. 1998; Xu *et al*. 1998). RpoS is expressed in *Ps. aeruginosa* (Fujita *et al*. 1994; Tanaka and Takahashi 1994; Latifi *et al*. 1996; Jørgensen *et al*. 1999; Suh *et al*. 1999) under stress conditions. RpoS has also been shown to be up-regulated in *Ps. aeruginosa* biofilms (Xu *et al*. 1999) and is highly expressed in the sputum of cystic fibrosis patients (Foley *et al*. 1999).

In an earlier report (Cochran *et al*. 2000), the use of alginate gel beads as an attachment substratum for biofilm formation was described. It was shown that the thin *Ps. aeruginosa* biofilms (10^5 cfu cm^{-2}) developed in this system were less susceptible to monochloramine and hydrogen peroxide than planktonic cells. Analysis of transport phenomena in the gel bead biofilm system showed that the decrease in biocide efficacy was not due to mass transport limitations. These thin biofilms did not inhibit penetration of the disinfectant as they were equivalent to less than a monolayer of cells. Cochran *et al*. (2000) hypothesized that the attached cells were physiologically altered to become less susceptible to oxidizing biocides.

The aim of the work reported here was to investigate the role of two alternative sigma factor genes, *algT* and *rpoS*, in the protection of biofilm cells against oxidative biocides. It was hypothesized that these genes are required to implement full biofilm resistance to hydrogen peroxide and monochloramine. *algT* and *rpoS* were chosen because they encode for sigma factors that are known to protect the cell against environmental stresses and because there are preliminary indications that these genes may be involved in biofilm phenomena.

MATERIALS AND METHODS

Bacterial strains, media and enumeration

The bacterial strains used in this study are listed in Table 1. PAO1 is a standard strain of *Ps. aeruginosa*. SS24 has the genotype *rpoS*::aacCl (Suh *et al*. 1999) and is therefore $RpoS^-$. PAO6852 has a tetracycline gene inserted into *algT*, thus inactivating this gene. *Escherichia coli* DH10B (Gibco) was routinely used as the host for cloning and harbouring plasmids. Modified R2A (APHA *et al*. 1995), without K_2HPO_4 and starch and with the addition of 3·4 mmol l^{-1} $CaCl_2.2H_2O$, was used as growth medium for all disinfection experiments. For molecular techniques, Luria broth (LB) (10 g tryptone, 5 g yeast extract, 5 g NaCl l^{-1}), alone or supplemented with ampicillin (100 μg ml^{-1}), carbenicillin (300 μg ml^{-1}) or gentamicin (20 μg ml^{-1}), was used. Enumeration of bacteria was performed by serial dilution in phosphate-buffered saline (pH 7·5) (PBS) (US Food and Drug Administration 1984) followed by drop-plating 10 μl drops (Miles and Misra 1938; Hoben and Somasegaran 1982) onto R2A plates (Difco).

Preparation of disinfectants

Monochloramine solution was prepared as previously described by Chen *et al*. (1993) and hydrogen peroxide solutions were prepared by diluting unstabilized hydrogen peroxide (30%) (Hach; ACS grade). Both monochloramine and hydrogen peroxide were titrated prior to each experiment to determine their concentrations. Monochloramine was titrated using a Hach amperometric titrator (Hach, Loveland, CO, USA). To determine hydrogen peroxide concentration, the method of Klothoff and Sandell (1952) was modified by titrating to a colourless endpoint using standardized 0·1 l^{-1} sodium thiosulphate.

Planktonic disinfection assay

An overnight culture of *Ps. aeruginosa* was sub-cultured into fresh R2A to a final cell concentration between 10^7 and 10^8

Table 1 Bacterial strains and plasmids

Strains or plasmid	Relevant properties	Source of reference
E. coli		
DH10B	Wild type	Gibco BRL
Ps. aeruginosa		
PAO1	Wild type	(Holloway 1955)
PAO6852	PAO1 *alg*T::Tc^r	(Martin *et al.* 1994)
SS24	PAO1 *rposS101*::*aacCI*	(Suh *et al.* 1999)
SS24 (pUCP19)	*rpoS*$^-$; SS24 carrying pUCP19	This work
SS24 (pSS32)	Wild type; SS24 carrying pSS32	This work
Plasmids		
PDB19R	PTZ19R containing a 1·8-kb *Kpn*I-*Hind*III fragment of *rpoS*	(Tanaka and Takahashi 1994)
PUCP19	Ap^r *lacZ*	(Schweizer 1991)
pSS32	pUCP19 containing *rpoS*	This work

cfu ml^{-1} and incubated for 4 h. The exponentially-growing culture of *Ps. aeruginosa* was homogenized with a tissue homogenizer for 1 min on ice. The cells were then diluted to 10^6 cfu ml^{-1} in phosphate-buffered water (pH 7) (PBW) (APHA *et al.* 1995). Monochloramine stock solution was added to the PBW to attain a final concentration of 2 mg l^{-1}. Cells were sampled at timed intervals over a 10 min period from the start of the experiment. Cells were removed from the monochloramine and diluted into PBS containing sodium thiosulphate (1 mmol l^{-1} final concentration) to neutralize the monochloramine. Total remaining chlorine in the reaction vessel was determined using the Hach amperometric titrator. Disinfection experiments were also performed with 600 mg l^{-1} hydrogen peroxide. Cells were sampled every 10 min for 60 min. The reaction was stopped using 4·12 mmol l^{-1}, final concentration, of sodium thiosulphate in PBS. Final hydrogen peroxide concentrations were determined by titration with sodium thiosulphate as described above.

As controls, planktonic cells were sub-cultured in PBW (10^6 cfu ml^{-1}) without the addition of an antimicrobial agent and sampled at the start and end of the disinfection assay. For all disinfection experiments, cells were serially diluted in PBS and enumerated by the drop plate method, as described above.

Alginate bead biofilm formation and disinfection

Cells were attached to alginate gel beads as described previously by Cochran *et al.* (2000). Biofilms were grown for 24 and 48 h before being exposed to oxidative biocides. Preparation of biocides and disinfection assay experiments were the same as the procedures used by Cochran *et al.* (1998). Biofilms were exposed to 2 mg l^{-1} monochloramine for 10 min and to 600 mg l^{-1} hydrogen peroxide for 60 min. Beads were removed at timed intervals and biocide was neutralized with sodium thiosulphate. Beads were dissolved in citrate buffer (Cochran *et al.* 2000) and cells were enumerated by drop-plating, as described above.

Glass slide biofilm formation and disinfection

Pseudomonas aeruginosa biofilms were grown on glass slides in a biofilm apparatus described by Cargill *et al.* (1992). Biofilms were grown as described by Cochran *et al.* (2000) for 24 h in conditions similar to the bead biofilms. Disinfection of the glass slides was carried out in a clean sterile jar to eliminate the oxidant demand associated with excessive organic matter. The slides were removed at timed intervals and placed into PBW containing sodium thiosulphate to neutralize the biocide. Treated slides were scraped into PBS and cells were homogenized and enumerated on R2A plates.

Calculation of disinfection rate coefficients

The model used to interpret disinfection rates was

$$dX/dt = -k_b CX$$

where the change in cell density with time is related to the disinfection coefficient, k_b, biocide concentration, C, and viable cell density, X. Raw disinfection rates were determined using the least squares method to calculate a regression line through the data consisting of the natural log of cfu cm^{-2} *vs* time. A slope with its standard error was calculated, where the slope was the disinfection rate for that experiment. The disinfection rate coefficient was determined by dividing the raw disinfection rate (min^{-1}) by the average biocide concentration (mg l^{-1}) to normalize against variations in dis-

infectant concentrations (Cochran *et al.* 2000). This coefficient has units of $l\ mg^{-1}\ min^{-1}$.

DNA manipulations, transformations and conjugations

Standard molecular biological techniques were used for DNA manipulation (Maniatis *et al.* 1982; Ausbel *et al.* 1987). Plasmids were purified with QIAprep spin miniprep columns made by Qiagen (Santa Clarita, CA, USA). DNA fragments were excised from agarose gels and purified using the Qiaex II DNA gel extraction system (Qiagen) according to the manufacturer's instructions. Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs, Gibco BRL or Boehringer Mannheim. Standard electroporation procedure was used for *E. coli* using the *E. coli* Gene Pulser by Bio-Rad (Hercules, CA, USA). For *Ps. aeruginosa*, electroporation was performed as previously decribed (Suh *et al.* 1999).

Construction of a *Ps. aeruginosa rpoS* complementing plasmid clone and complementation of SS24

A 1·9 kb *EcoR*I-*Hind*III fragment which carries the complete *Ps. aeruginosa rpoS* gene was acquired from plasmid pDB19R (Tanaka and Takahashi 1994) and cloned into the *Ps. aeruginosa–E. coli* shuttle vector pUCP19 (Schweizer 1991) to generate pSS32. The orientation of the *rpoS* in pSS32 dictates that *rpoS* expression occurs from the native promoter and not from the *lac* promoter carried on pUCP19. pSS32 was then introduced into SS24 and complementation of the *rpoS* mutant phenotype was determined by immunoblot analysis.

SDS–PAGE and immunoblot analysis

PAO1, SS24, SS24(pSS32) and SS24(pUCP19) were grown in LB overnight at 37 °C and sub-cultured until cell densities reached stationary phase, corresponding to an $O.D._{600}$ of 2·2. A 1 ml sample of cells was harvested and pelleted at 13 500 *g* for 5 min. Pellets were resuspended in 100 μl sterile H_2O and 100 μl Laemmli sample buffer (Laemmli 1970) were added. Samples were stored at –20 °C until use. Separation of proteins by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the Laemmli method (Laemmli 1970) with 3% stacking gels and 10% resolving gels. Proteins were transferred to nitrocellulose filter paper. Immunostaining was carried out using a 1 : 5000 dilution of polyclonal anti-RpoS (Zgurskaya *et al.* 1997), a gift from Dr Matin, followed by incubation with a 1 : 6000 dilution peroxidase-conjugated antirabbit IgG (Bio-Rad). RpoS-related protein bands were detected with a Bio-Rad Fluor-S Multiimager (Bio-Rad Labs, Hercules, CA, USA) according to the manufacturer's directions.

Statistical analysis

All statistical analyses were performed using Minitab Release 11.12 (Minitab, INC). *P*-values were calculated using the null hypothesis that the two sets of disinfection rate coefficients were the same. A *P*-value less than 0·05 indicated that the two sets were statistically different at the 95% confidence level.

RESULTS

Gel bead biofilm disinfection

Planktonic disinfection coefficients for monochloramine experiments for all strains fell within a tight cluster of values from 0·46 to 0·61 $l\ mg^{-1}\ min^{-1}$ (Fig. 1). These values were significantly higher than the rates obtained for biofilms grown on alginate gel beads, whose disinfection rate coefficients ranged from 0·19 to 0·29 $l\ mg^{-1}\ min^{-1}$ ($P \leqslant 0{\cdot}015$).

Biofilm accumulation on alginate gel beads was sparse, with a mean areal cell density of untreated biofilms of $3{\cdot}7 \pm 0{\cdot}30$ log cfu cm^{-2} after 24 h and $3{\cdot}8 \pm 0{\cdot}08$ cfu cm^{-2} after 48 h of growth.

Biofilm susceptibility to hydrogen peroxide was clearly strain dependent. While the planktonic disinfection rate coefficients for all strains remained in a tight cluster with values ranging from $3{\cdot}1 \times 10^{-4}$–$3{\cdot}5 \times 10^{-4}$ $l\ mg^{-1}\ min^{-1}$ (Fig. 2), PAO6852 (*algT*$^-$) and SS24 (*rpoS*$^-$) 24-hour biofilms were significantly more susceptible to hydrogen peroxide than was the wild type PAO1 ($P \leqslant 0{\cdot}03$). PAO6852 and SS24 biofilm disinfection rate coefficients were comparable with planktonic disinfection rate coefficients ($P \geqslant 0{\cdot}15$). After 48 h of biofilm growth, PAO6852 became less susceptible, with disinfection

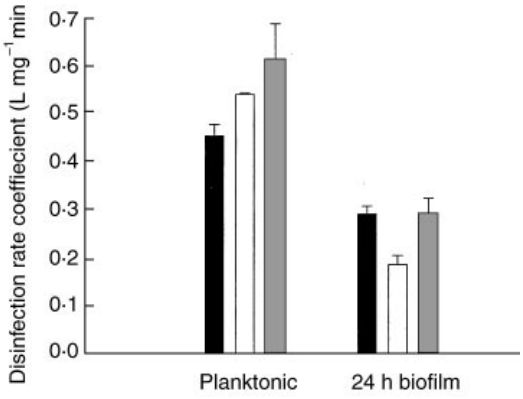

Fig. 1 Mean disinfection rate coefficients of *Pseudomonas aeruginosa* strains, planktonic and 24-hour-old bead biofilms, using 2 $mg\ l^{-1}$ monochloramine. Biofilms were grown on alginate gel beads. Error bars represent standard error of the mean. (■), PAO1; (□), PAO6852 (*algT*$^-$); (▩), SS24 (*rpoS*$^-$)

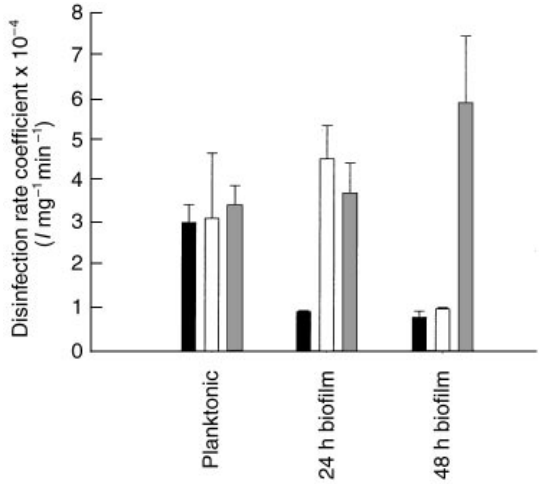

Fig. 2 Mean disinfection rate coefficients of *Pseudomonas aeruginosa* strains, planktonic and 24- and 48-hour-old bead biofilms, using 600 mg l^{-1} hydrogen peroxide. Biofilms were grown on alginate gel beads. Error bars represent standard error of the mean. (■), PAO1; (□), PAO6852 (*algT*⁻); (▩), SS24 (*rpoS*⁻)

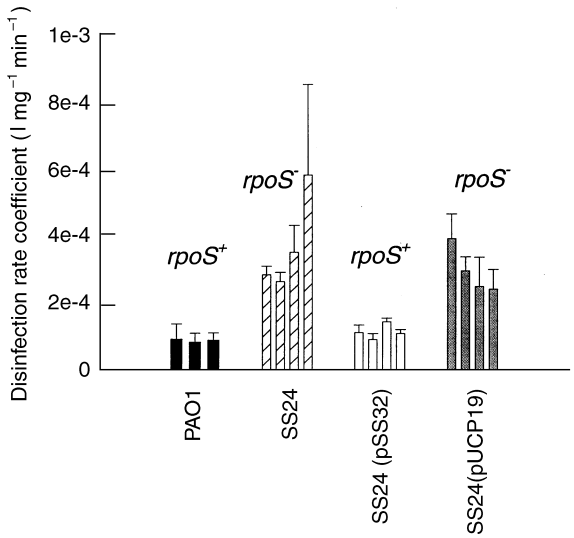

Fig. 3 Disinfection rate coefficients of *Pseudomonas aeruginosa* strains, with and without *rpoS*, using 600 mg l^{-1} hydrogen peroxide. Biofilms were grown on alginate gel beads. Error bars represent standard error of the disinfection rate coefficients

rate coefficients statistically the same as 48-hour-old PAO1 biofilms ($P = 0{\cdot}70$). However, SS24 remained highly susceptible to hydrogen peroxide, with coefficients comparable with planktonic wild type disinfection rate coefficients ($P = 0{\cdot}51$).

RpoS complementation and Western blot analysis

To confirm the findings from Fig. 2 that *rpoS* expression protects biofilm from hydrogen peroxide disinfection, *Ps. aeruginosa* strain SS24(pSS32) was made. This complement strain was constructed by electroporating pSS32 into SS24, therefore restoring a wild type genotype. Hydrogen peroxide alginate gel bead biofilm disinfection experiments were repeated with SS24(pSS32) and its vector control SS24(pUCP19). SS24(pUCP19) is SS24 containing the vector, pUCP19, without the *rpoS* insert, and this strain has an *rpoS*⁻ genotype. There were no differences between the strains in the planktonic experiments (data not shown). SS24(pSS32) biofilms of 24 hours old were resistant to hydrogen peroxide, with coefficients similar to PAO1 24-hour-old biofilms ($P = 0{\cdot}12$) (Fig. 3); 24-hour biofilms experiments with the vector control, SS24(pUCP19), resulted in disinfection rate coefficients comparable with SS24 and planktonic coefficients ($P = 0{\cdot}88$).

Antibodies raised against *E. coli* RpoS protein were used to probe a Western blot of stationary phase cell extracts of PAO1, SS24, SS24(pSS32) and SS24(pUCP19). Figure 4 shows that extracts from PAO1 and SS24(pSS32) each contained a specific protein band detected by the *E. coli* RpoS

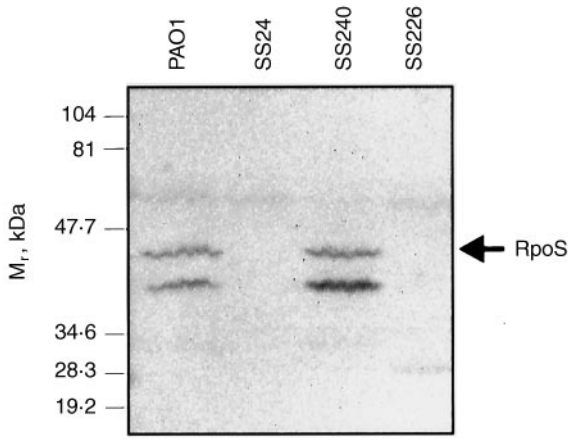

Fig. 4 Immunoblot analysis of proteins of wild type, PAO1; *rpoS* mutant, SS24; *rpoS* complement strain, SS240; and vector control, SS226

antibody. The extracts of SS24 and SS24(pUCP19) did not contain this band. The second lower molecular weight band seen in PAO1 and SS24(pSS32) was believed to be a degradation product of RpoS. When log phase, late log phase, transition phase, stationary phase planktonic PAO1 cultures were used for Western blot analysis, the lower band appeared only in the stationary phase samples, not in the log, late log or transition phase cell extracts (data not shown).

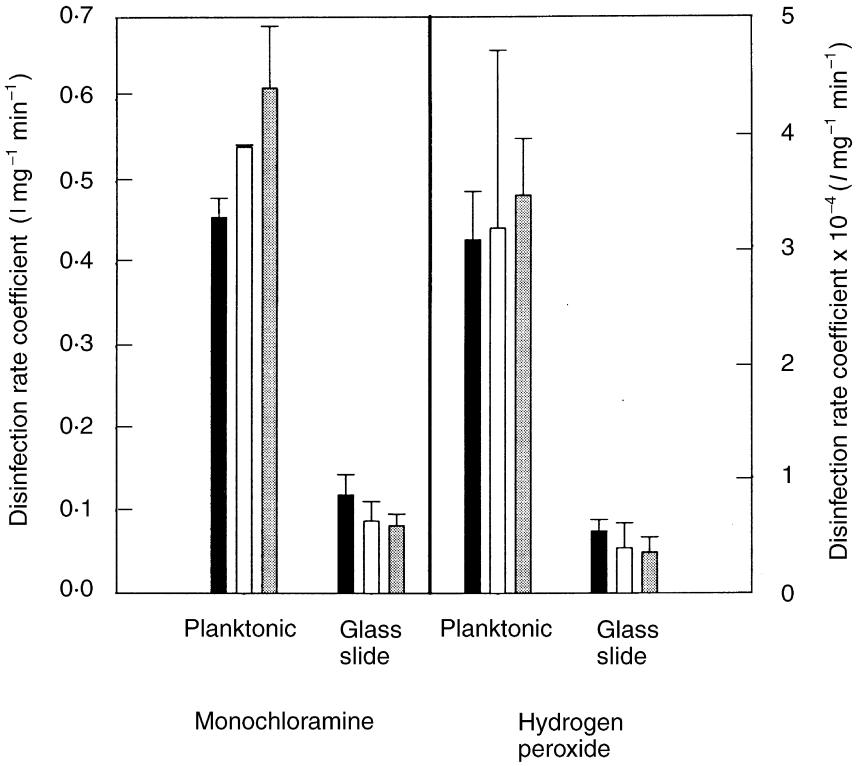

Fig. 5 Mean disinfection rate coefficients of *Pseudomonas aeruginosa* strains: (■), PAO1, (□), PAO6852 (*algT*$^-$), (▦), SS24 (*rpoS*$^-$) using 2 mg l^{-1} monochloramine (left axis) and 600 mg l^{-1} hydrogen peroxide (right axis). Biofilms were grown on glass coupons for 24 h in conditions similar to alginate gel beads. Error bars represent standard error of the mean

Glass slide biofilm disinfection

Thick biofilms grown on glass slides exhibited resistance to both biocides (Fig. 5). The glass slides were more heavily colonized than the alginate gel beads. The slides had an average of 7·6 ± 0·27 log cfu cm^{-2}. The 24-hour glass slide biofilms of all strains had similar disinfection rates when challenged with monochloramine and hydrogen peroxide. Disinfection rate coefficients of 24-hour glass biofilms ranged from 0·082 to 0·121 mg^{-1} min^{-1} with monochloramine, and 3·6 × 10^{-5}–5·6 × 10^{-5} l mg^{-1} min^{-1} with hydrogen peroxide. Unlike disinfection of biofilms growing on alginate gel beads, there were no observed differences in disinfection rate coefficients when using PAO6852 and SS24 with hydrogen peroxide on glass slides ($P > 0·0089$). These strains were as resistant to hydrogen peroxide as PAO1.

DISCUSSION

Pseudomonas aeruginosa susceptibility to both monochloramine and hydrogen peroxide was significantly reduced when bacteria were grown as biofilms compared with the susceptibility of freely suspended cells. Reduced biofilm susceptibility was manifested in both thin biofilms grown on alginate gel beads and in thicker biofilms grown on glass slides. The ratio of the disinfection rate coefficient measured for planktonic cells to that measured for biofilms, which is a quantitative measure of biofilm resistance, was approximately 1·6–3·9 for monochloramine and 3·9–5·8 for hydrogen peroxide. It is concluded that both biofilm models used in this work capture the phenomenon of reduced biofilm susceptibility and are therefore acceptable experimental systems for investigating mechanisms of biofilm antimicrobial resistance.

In thin alginate gel bead biofilms, a statistically significant contribution of both AlgT and RpoS to resistance to hydrogen peroxide could be demonstrated. However, the protection afforded by AlgT was transient; it was significant in 24 hour-old biofilms but could not be discerned in 48 hour-old biofilms. RpoS provided peroxide protection for at least 48 h. The role of RpoS protection to peroxide was further supported by restoration of the wild type phenotype with a complementing plasmid in the mutant strain. Complemented strains were once again resistant to hydrogen peroxide. These findings represent one of the first demonstrations of a link between a specific gene and antimicrobial resistance in a biofilm.

However, the protection afforded by AlgT and RpoS in biofilms was very limited. No contribution of either sigma factor gene to biofilm reduced susceptibility could be detected when gel bead biofilms were challenged with monochloramine. Neither did these genes confer protection to

either biocide in thicker biofilms developed on glass slides. In these thicker biofilms, poor penetration of the antimicrobial agents probably contributes to biofilm resistance (Holyle *et al.* 1992; Stewart 1996, 1999; Xu *et al.* 1996). It is concluded that the AlgT and RpoS do not mediate generic reduced antimicrobial susceptibility of *Ps. aeruginosa* biofilms.

These results by no means rule out a genetic basis for biofilm reduced susceptibility to antimicrobial agents. They do suggest that it may be more productive to implement fresh biofilm-based screens for important biofilm genes than to test existing mutants, which have all been derived based on planktonic methods. Evidence for the genetic determination of biofilm development is growing (Davies *et al.* 1993; Yun *et al.* 1994; Heilmann *et al.* 1996; Christensen *et al.* 1998; Moller *et al.* 1998; Stickler *et al.* 1998; Xu, 1999), so it seems only a matter of time before key genes involved in protecting biofilms from antimicrobial challenges are identified.

ACKNOWLEDGEMENTS

This work was supported by the Center for Biofilm Engineering at Montana State University, a National Science Foundation-sponsored Engineering Research Center (co-operative agreement ECD-8907039); partial funding was provided by the Cystic Fibrosis Foundation postdoctoral fellowship SUH96F0. The authors thank Karen Xu for assistance with the immunoblot technology and Brian VanderVen for technical support.

REFERENCES

American Public Health Association (APHA), American Water Works Association and Water and Environment Federation (1995) *Standard Methods for the Examination of Water and Wastewater*. Washington, D.C.: American Public Health Association.

Ausbel, F.M., Brent, R., Kingston, R.E., Seidman, J.G., Smith, J.A. and Struhl, R. (1987) *Current Protocols in Molecular Biology*. New York: Green Publishing Associates and John Wiley-Interscience.

Boyd, A. and Chakrabarty, A.M. (1995) *Pseudomonas aeruginosa* biofilms: role of the alginate exopolysacchride. *Journal of Industrial Microbiology* **15,** 162–168.

Brown, M.R.W. and Gilbert, P. (1993) Sensitivity of biofilms to antimicrobial agents. *Journal of Applied Bacteriology* **74,** 87S–97S.

Cargill, K.L., Pyle, B.H., Sauer, R.L. and McFeters, G.A. (1992) Effects of culture conditions and biofilm formation on the iodine susceptibility of *L. pneumophila*. *Canadian Journal of Microbiology* **38,** 423–429.

Chen, C.-I., Griebe, T. and Characklis, W.G. (1993) Biocide action of monochloramine on biofilm systems of *Pseudomonas aeruginosa*. *Biofouling* **7,** 1–17.

Christensen, B.B., Sternberg, C., Anderson, J.B. *et al.* (1998) Establishment of new genetic traits in a microbial biofilm community. *Applied and Environmental Microbiology* **64,** 2247–2255.

Cochran, W.L., McFeters, G.A. and Stewart, P.S. (2000) Reduced susceptibility of thin *Pseudomonas aeruginosa* biofilms to hydrogen peroxide and monochloramine. *Journal of Applied Microbiology* **88,** 22–30.

Davies, D.G., Chakrabarty, A.M. and Geesey, G.G. (1993) Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology* **59,** 1181–1186.

de Beer, D., Stoodley, P., Roe, F. and Lewandowski, Z. (1994) Effects of biofilm structure on oxygen distribution and mass transport. *Biotechnology and Bioengineering* **43,** 1131–1138.

Demple, B., Halbrook, J. and Linn, S. (1983) *Escherichia coli xth* mutants are hypersensitve to hydrogen peroxide. *Journal of Bacteriology* **153,** 1079–1082.

Foley, I., Marsh, P., Wellington, E.M.H., Smith, A.W. and Brown, M.R.W. (1999) General stress response master regulator *rpo*S is expressed in human infection: a possible role in chronicity. *Journal of Antimicrobial Chemotherapy* **43,** 164–165.

Fujita, M., Tanaka, K., Takahashi, H. and Amemura, A. (1994) Transcription of the principal sigma-factor gene, *rpoD* and *rpoS*, in *Pseudomonas aeruginosa* is controlled according to the growth phase. *Molecular Microbiology* **13,** 1071–1077.

Heilmann, C., Schweitzer, O., Gerke, C., Vanittanakom, N., Mack, D. and Gotz, F. (1996) Molecular basis of intercellular adhesion in biofilm-forming *Staphyloccoccus epidermidis*. *Molecular Microbiology* **20,** 1083–1091.

Hershberger, C., Ye, R., Parsek, M., Xie, Z. and Chakrabarty, A. (1995) The *algT* (*algU*) gene of *Pseudomonas aeruginosa*, a key regulator involved in alginate biosynthesis, encodes an alternative σ factor (σ^E). *Proceedings from National Academy of Science, USA* **92,** 7941–7945.

Hoben, H.J. and Somasegaran, P. (1982) Comparison of the pour, spread, and drop plate methods for enumeration of *Rhizobium* spp. in inoculants made for presterilized peat. *Applied and Environmental Microbiology* **44,** 1246–1247.

Holloway, B.W. (1955) Genetic recombination in *Pseudomonas aeruginosa*. *Journal of General Microbiology* **13,** 572–581.

Holyle, B.D., Alcantara, J. and Costerton, J.W. (1992) *Pseudomonas aeruginosa* biofilm as a diffusion barrier to piperacillin. *Antimicrobial Agents and Chemotherapy* **36,** 2054–2056.

Huang, C.-T., Xu, K.D., McFeters, G.A. and Stewart, P.S. (1998) Spatial patterns of alkaline phosphatase expression within bacterial colonies and biofilm in response to phosphate starvation. *Applied and Environmental Microbiology* **64,** 1526–1531.

Huang, C.-T., Yu, F.P., McFeters, G.A. and Stewart, P.S. (1995) Nonuniform spatial patterns of respiratory activity within biofilms during disinfection. *Applied and Environmental Microbiology* **61,** 2252–2256.

Ivanova, A., Miller, C., Glinsky, G. and Eisenstark, A. (1994) Role of *rpoS* (*katF*) in *oxyR*-independent regulation of hydroperoxidase I in *Escherichia coli*. *Molecular Microbiology* **12,** 571–578.

Jørgensen, F., Bally, M., Chapon-Herve, V. *et al.* (1999) RpoS-dependent stress tolerance in *Pseudomonas aeruginosa*. *Microbiology* **145,** 835–844.

Klothoff, I.M. and Sandell, E.B. (1952) *Textbook of Quantitative Inorganic Analysis*. New York: The McMillan Company.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* **227,** 680–685.

Lange, R. and Hengge-Aronis, R. (1991) Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Molecular Microbiology* **5,** 49–59.

Latifi, A., Foglino, M., Tanaka, K., Williams, P. and Lazdunski, A. (1996) A hierarchical quorum-sensing cascade in *Pseudomonas aeruginsa* links the transcriptional activators LasR and RhlR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Molecular Microbiology* **21,** 1137–1146.

LeMagrex, E., Brisset, L., Jacquelin, L.F., Carquin, J. and Bonnaveiro, N. (1994) Susceptibility to antibacterials and compared metabolism of suspended bacteria versus embedded bacteria in biofilms. *Colloids and Surfaces B: Biointerfaces* **2,** 89–95.

Loewen, P.C., Switala, J. and Triggs-Raine, B.L. (1985) Catalases HPI and HPII in *Escherichia coli* are induced independently. *Archives of Biochemistry and Biophysics* **243,** 144–149.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Martin, D.W., Schurr, M.J. and Deretic, V. (1994) Analysis of promoters controlled by the putative sigma factor AlgU regulating conversion to mucoidy in *Pseudomonas aeruginosa*: Relationship to σ^E and stress response. *Journal of Bacteriology* **176,** 6688–6696.

Miles, A.A. and Misra, S.S. (1938) The estimation of the bactericidal power of blood. *Journal of Hygiene* **38,** 732–749.

Moller, S., Sternberg, C., Anderson, J.B. *et al.* (1998) *In situ* gene expression in mixed-culture biofilms: Evidence of metabolic interactions between community members. *Applied and Environmental Microbiology* **64,** 721–732.

Nichols, W.W., Dorrington, S.M., Slack, M.P.E. and Walmsley, H.L. (1988) Inhibition of tobramycin diffusion by binding to alginate. *Antimicrobial Agents and Chemotherapy* **32,** 518–523.

Nichols, W.W., Evans, M.J., Slack, M.P.E. and Walmsley, H.L. (1989) The penetration of antibiotics into aggregates of mucoid and non-mucoid *Pseudomonas aeruginosa*. *Journal of General Microbiology* **135,** 1291–1303.

Nickel, J.C., Rusesla, I., Wright, J.B. and Costerton, J.W. (1985) Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. *Antimicrobial Agents and Chemotherapy* **27,** 619–624.

Sanderson, S.S. and Stewart, P.S. (1997) Evidence of bacterial adaptation to monochloramine in *Pseudomonas aeruginosa* biofilms and evaluation of biocide action model. *Biotechnology and Bioengineering* **56,** 201–209.

Schurr, M.J., Yu, H., Martinez-Salazar, J.M., Boucher, J.C. and Deretic, V. (1996) Control of AlgU, a member of the σ^E- like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. *Journal of Bacteriology* **178,** 4997–5004.

Schweizer, H.P. (1991) *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. *Gene* **97,** 109–121.

Srinivasan, R., Stewart, P.S., Griebe, T., Chen, C.-I. and Xu, X. (1995) Biofilm parameters influencing biocide efficacy. *Biotechnology and Bioengineering* **46,** 553–560.

Stewart, P.S. (1996) Theoretical aspects of antibiotic diffusion into microbial biofilms. *Antimicrobial Agents and Chemotherapy* **40,** 2517–2522.

Stewart, P.S. (1998) A review of experimental measurements of effective diffusive permeabilities and effective diffusion coefficients in biofilms. *Biotechnology and Bioengineering* **59,** 261–272.

Stewart, P.S. and Raquepas, J.B. (1995) Implications of reaction-diffusion theory for the disinfection of microbial biofilms by reactive antimicrobial agents. *Chemical Engineering Science* **50,** 3099–3104.

Stewart, P.S., Roe, F., Rayner, J. *et al.* (1999) Effect of catalase on hydrogen peroxide penetration into *Pseudomonas aeruginosa* biofilms. *Applied and Environmental Microbiology* in press.

Stickler, D.J., Morris, N.S., McLean, R.J.C. and Fuqua, W.C. (1998) Biofilm on indwelling urethral catheters produce quorum-sensing signal molecules *in situ* and *in vitro*. *Applied and Environmental Microbiology* **64,** 3486–3490.

Suh, S.-J., Silo-Suh, L.A., Woods, D.E., Hassett, D.J., West, S.E.H. and Ohman, D.E. (1999) Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **181,** 3890–3897.

Tanaka, K. and Takahashi, H. (1994) Cloning, analysis and expression of an *rpoS* homologue gene from *Pseudomonas aeruginosa* PAO1. *Gene* **150,** 81–85.

US Food and Drug Administration (1984) *Bacteriological Analytical Manual*. Washington, D.C.: US Food and Drug Administration.

Wentland, E.J., Stewart, P.S., Huang, C.-T. and McFeters, G.A. (1996) Spatial variations in growth rate within *Klebsiella pneumoniae* colonies and biofilm. *Biotechnological Progress* **12,** 316–321.

Xie, Z., Hershberger, C., Shankar, S., Ye, R. and Chakrabarty, A. (1996) Sigma factor–anti-sigma factor interaction in alginate synthesis: inhibition of AlgT by MucA. *Journal of Bacteriology* **178,** 4990–4996.

Xu, K.D. Personal communication.

Xu, X., Stewart, P.S. and Chen, X. (1996) Transport limitation of chlorine disinfection of *Pseudomonas aeruginosa* entrapped in alginate beads. *Biotechnology and Bioengineering* **49,** 93–100.

Xu, K.D., Stewart, P., Xia, F., Huang, C.-T. and McFeters, G.A. (1998) Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. *Applied and Environmental Microbiology* **64,** 4035–4039.

Yu, H., Schurr, M.J. and Deretic, V. (1995) Functional equivalence of *Escherichia coli* σ^E and *Pseudomonas aeruginosa* AlgU: *E. coli rpoE* restores mucoidy and reduces sensitivity to reactive oxygen intermediates in *algU* mutants of *Ps. aeruginosa*. *Journal of Bacteriology* **177,** 3259–3268.

Yun, C., Ely, B. and Smit, J. (1994) Identification of genes affecting production of the adhesive holdfast of a marine caulobacter. *Journal of Bacteriology* **176,** 769–803.

Zgurskaya, H.I., Keyhan, M. and Matin, A. (1997) The σ^s level in starving *Escherichia coli* cells increases solely as a result of its increased stability, despite decreased synthesis. *Molecular Microbiology* **24,** 643–651.