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Short Communication

Characterisation of biofilm formation by a *Streptococcus suis* meningitis isolate

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Abstract

Biofilm formation by a strain of *Streptococcus suis* serotype 2 isolated from a case of meningitis in pigs was characterised. Using a polystyrene microtitre plate assay, *S. suis* 95-8242 produced a dense biofilm when glucose, fructose or sucrose was used as the carbohydrate source, whereas no biofilm formed in the presence of lactose. Polysaccharide production by the biofilm-forming strain was demonstrated by the Congo red agar assay. Transmission electron microscopy revealed that bacterial cells were surrounded by a thick layer of polycationic ferritin-labelled material. *S. suis* 95-8242 was more resistant to both penicillin G and ampicillin in biofilms than in planktonic cultures on the basis of minimal inhibitory and minimal bactericidal concentrations.

Keywords: *Streptococcus suis* type 2; Biofilm; Carbohydrates; Antibiotic sensitivity

Streptococcus suis is a major pathogen of pigs worldwide that colonises the respiratory tract and causes septicaemia, meningitis and endocarditis (Higgins and Gottschalk, 1999). Thirty-five serotypes (1–34 and 1/2) have been identified, of which serotype 2 is the most common isolate from diseased pigs. *S. suis* binds to extracellular matrix proteins, including fibronectin and collagen (Esgleas et al., 2005), as well as to endothelial and epithelial cells (Charland et al., 2000; Benga et al., 2004), but the mechanisms by which the bacterium invades and infects the host are unclear (Segura and Gottschalk, 2004).

The ability of bacteria to colonise host tissues is a critical step in the initiation of infection. Biofilms are defined as communities of microorganisms that adhere to each other and to solid surfaces and are enclosed in an extracellular matrix composed of materials from both the microorganisms and the environment. The formation of biofilms by microorganisms is a mechanism that allows them to become persistent colonisers, resist clearance by the host immune system, enhance resistance to antibiotics and exchange genetic materials (Donlan and Costerton, 2002). In this study, biofilm formation and antimicrobial resistance by a strain of *S. suis* isolated from a case of meningitis in pigs was characterised.

S. suis 95-8242 isolated from a pig with meningitis was cultured in broth containing (w/v) 0.5% glucose, 2% peptone, 0.3% K_2HPO_4, 0.2% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.002% $MnSO_4 \cdot 6H_2O$ and 0.5% NaCl. An overnight culture was diluted to an optical density of 0.1 at 655 nm (OD_{655}) and 200 μL were added to wells of a 96-well tissue culture plate (Sarstedt). After incubation for 24 h at 37 °C, medium and free-floating bacteria were removed by aspiration and the wells were washed three times with 50 mM phosphate-buffered saline (pH 7.2; PBS). The biofilms were stained with 0.04% crystal violet (100 μL) for 10 min. The wells were washed three times with PBS to remove unbound crystal violet dye and dried for 2 h at 37 °C. After adding 100 μL 95% (v/v) ethanol to each well, the plate was shaken for 10 min to release the stain from the biofilms and

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the absorbance at 550 nm (A_{550}) was recorded. Wells with sterile broth medium served as controls. Biofilm formation was determined in the presence of 0.25%, 0.5%, 1% and 2% glucose, as well as 0.5% fructose, lactose and sucrose. All biofilm assays were run in triplicate and the means ± standard deviations of two independent experiments were calculated.

Fig. 1 shows the kinetics of biofilm formation by *S. suis* 95-8242 in the polystyrene microtitre plate assay. The biofilm was formed completely after 6 h incubation and the phenotype was maintained through up to 15 subcultures in broth medium (data not shown), suggesting that the capacity to form a biofilm was a stable characteristic. Additional strains of *S. suis*, including one serotype 1 (S428), one serotype 1/2 (2651), 21 serotype 2 (166, 24, 31533, 770 297, 770 353, 89-5046, 89-999, 90-1330, 90-2741-7, 94-3037, 94-623, 98-3473-5, 98-3634, AAH4, D282, EA 0891/90, JL590, LM 90-559, Reims, S735, TD10), one serotype 3 (4961), and one serotype 5 (Amy12C) strains, were tested for their ability to form biofilms. A significant biofilm was formed by strain Amy12C, whereas less extensive biofilms were produced by strains AAH4, 98-3473-5, and 98-3634 (Fig. 2).

Biofilm formation by *S. suis* 95-8242 was not influenced by the concentration of glucose (0.5–2%, data not shown). Using glucose, fructose or sucrose as the carbohydrate source had no effect on biofilm formation (Fig. 1). However, when lactose was used as the carbohydrate source, the biofilm produced by *S. suis* 95-8242 was markedly reduced, despite the fact that growth was unaffected. This suggests that lactose may not be an appropriate carbohydrate source for extracellular matrix production by this strain.

The structural architecture of the *S. suis* biofilm was examined by scanning electron microscopy. *S. suis* was inoculated as above (5 mL/dish) in 35 mm dishes containing a 10.5 mm × 22 mm plastic coverslip (Nunc). After 24 h incubation, medium and free-floating bacteria were removed. The biofilms were incubated overnight in fixation buffer (4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2), washed with 0.1 M cacodylate buffer pH 7.0 (3 × 20 min) and post-fixed for 90 min at room temperature in 1% osmic acid containing 2 mM potassium ferrocyanide and 6% sucrose in cacodylate buffer. Samples were dehydrated through a graded series of ethanol (50%, 70%, 95% and 100%), critical point dried, gold sputtered and examined using a JEOL JSM6360LV

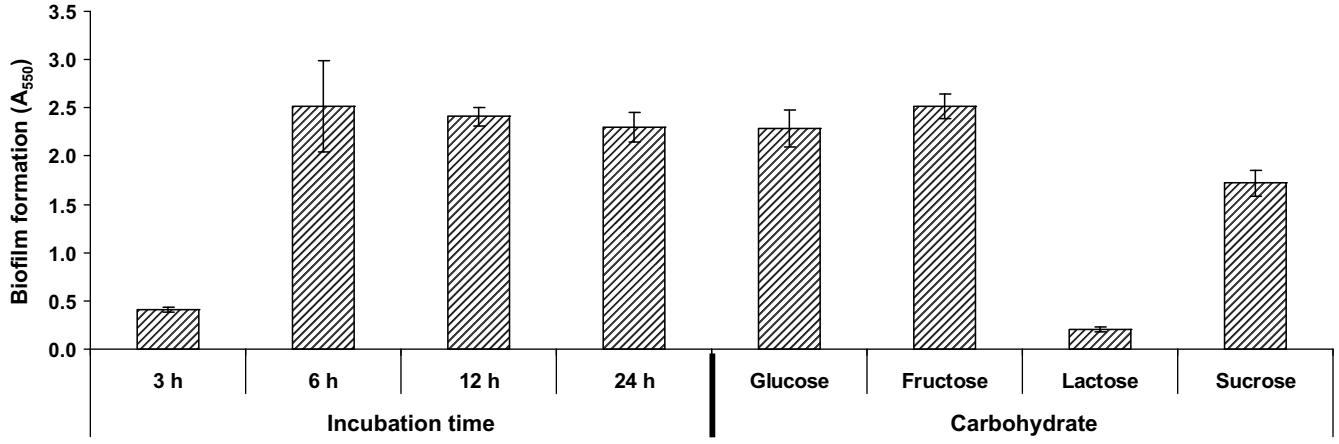

Fig. 1. Biofilm formation by *S. suis* 95-8242 determined by the polystyrene microtitre plate assay. Effect of incubation time and carbohydrates.

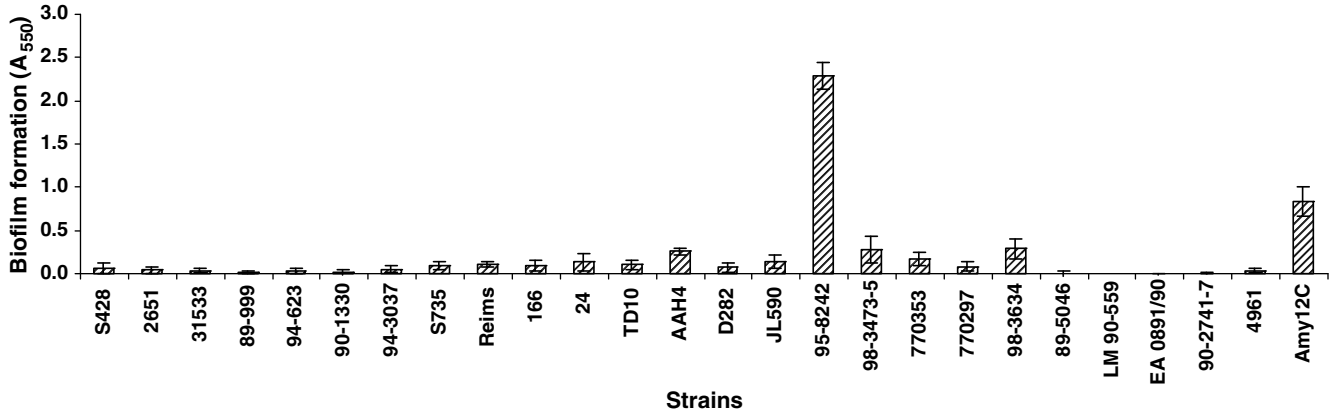

Fig. 2. Capacity of various strains of *S. suis* to form biofilms determined by the polystyrene microtitre plate assay.

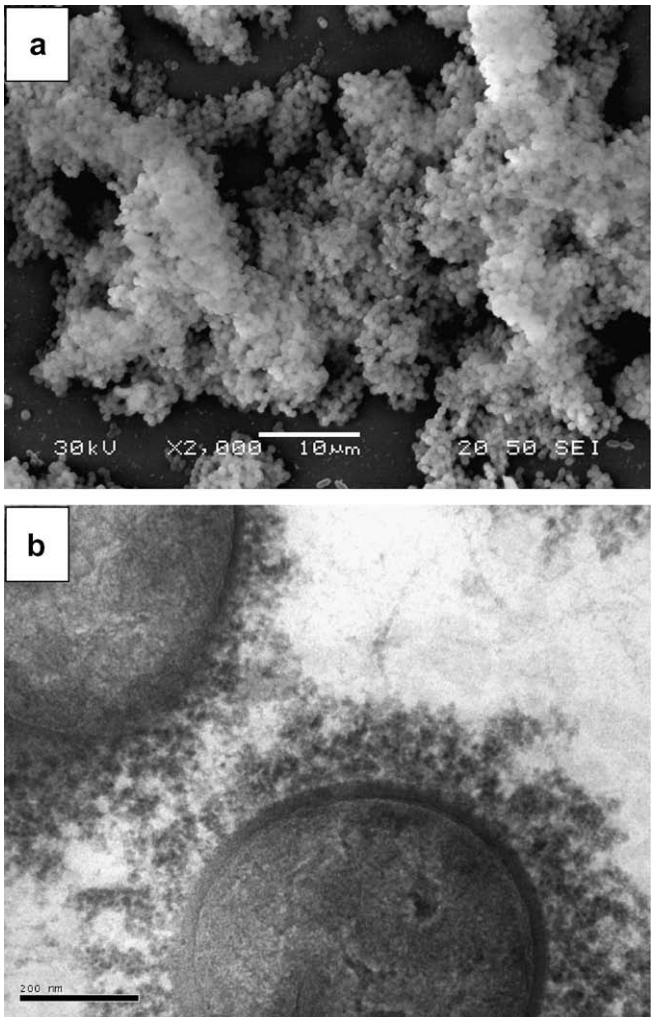

Fig. 3. Scanning electron micrograph of *S. suis* 95-8242 biofilm formed after 24 h growth (a). Transmission electron micrograph of thin sections of *S. suis* 95-8242 cells labelled with polycationic ferritin (b).

scanning electron microscope at 30 kV. Scanning microscopic examination of strain 95-8242 showed the capacity of this isolate to form a dense biofilm (Fig. 3a). Aggregates and microcolonies of *S. suis* almost completely covered the surface of the coverslip.

Slime production by *S. suis* was investigated using the Congo red agar (CRA) assay (Freeman et al., 1989). *S. suis* 95-8242 was demonstrated to be a slime-producing strain. The presence of material surrounding bacterial cells was investigated by transmission electron microscopy. Bacteria were grown in broth medium, harvested by centrifugation and washed once in PBS. The cells were fixed for 2 h at room temperature in 0.1 M cacodylate buffer (pH 7.0) containing 5% glutaraldehyde and 0.15% ruthenium red, then reacted with polycationic ferritin (1 mg/mL) and processed as described by Vanrobaeys et al. (1999). Thin sections were examined using a JEOL 1230 transmission electron microscope at 60 kV. Material surrounding the *S. suis* cells was distributed regularly and was approximately 200 nm thick (Fig. 3b). Labelling with polycationic ferritin suggests that the material is rich in acidic polysaccharides.

The minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) of penicillin G and ampicillin for planktonic *S. suis* 95-8242 were determined using microtitre plate assays. Two-fold serial dilutions of antibiotics were prepared in culture broth. Wells of microtitre plates, each containing 100 µL of medium, were inoculated with 100 µL of an overnight culture of *S. suis* 95-8242 diluted in fresh culture broth to obtain an OD_{655} of 0.2 and incubated at 37 °C for 24 h. The MIC was the lowest concentration of antibiotic for which no significant increase in OD_{655} was noted. To determine MBCs, 10 µL of culture was recovered from wells with no visible growth and spread on Todd Hewitt agar (Difco Laboratories) plates. The MBC was the lowest concentration of antibiotic at which no colonies grew on the agar medium.

The MICs and MBCs of biofilm *S. suis* 95-8242 were determined using a microtitre plate containing a 24 h preformed biofilm in each well. After aspiration of the culture supernatants, 200 µL of fresh culture broth containing two-fold serial dilutions of antibiotic were added to each well and the plate was incubated for 24 h at 37 °C. The biofilm was suspended by scraping and growth was estimated by recording the OD_{655} at the end of the 24 h incubation period. The MBCs of the biofilm-grown cells were determined by incubating the biofilm for 24 h with penicillin G or ampicillin, suspension in Todd Hewitt broth and spreading 10 µL on Todd Hewitt agar plates.

Table 1 lists the MICs and MBCs of penicillin G and ampicillin for *S. suis* 95-8242 grown in planktonic cultures and biofilms. *S. suis* 95-8242 grown in a biofilm was much more resistant to penicillin G and ampicillin than planktonic cells; the MICs and MBCs of the two antibiotics for biofilms were both >1000 µg/mL. *S. suis* AAH4, which is a low biofilm-producing strain, also had increased MBCs and, to a lesser extent, MICs of both antibiotics when grown as a biofilm, but this capacity was less than *S. suis* 95-8242.

The ability of several pathogenic microorganisms to form biofilms on host surfaces contributes to their virulence. In this study, we showed that a *S. suis* meningitis isolate is

Table 1
Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) of penicillin G and ampicillin for planktonic- and biofilm-grown *S. suis* 95-8242 and AAH4

Strain	Growth	Antibiotic			
		Penicillin G		Ampicillin	
		MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
95-8242	Planktonic	0.06	3.9	0.24	7.8
	Biofilm	>1000	>1000	>1000	>1000
AAH4	Planktonic	0.03	0.03	0.125	0.125
	Biofilm	1	16	1	32

Values are representative of six independent experiments.

capable of forming a dense biofilm. The formation of most bacterial biofilms depends on the production of an extracellular matrix containing secreted polysaccharides and proteins. Strain 95-8242 gave a positive result using the CRA plate assay, suggesting that the production of an extracellular matrix may be important for biofilm formation by this particular strain. Congo red stains polysaccharides containing contiguous β-(1→4)-linked D-glucopyranosyl units and β-(1→3)-D-glucans and has been used to identify exopolysaccharides in biofilms (Wood, 1980). The production of an extracellular matrix by *S. suis* was confirmed by transmission electron microscopy that showed a thick layer of polycationic ferritin-labelled material.

The biofilm-grown *S. suis* 95-8242 was demonstrated to be significantly more resistant to both penicillin G and ampicillin than planktonic cells. The mechanism of biofilm-associated antimicrobial resistance is multifactorial (Patel, 2005). Biofilms provide resistance to antibiotics because of their stable architecture, which restricts the penetration of antibiotics. In addition, bacteria within a biofilm multiply very slowly and therefore are much less susceptible to growth-dependent antimicrobial killing.

The ability of *S. suis* to form a biofilm may be a virulence factor that contributes to the establishment of infections, such as meningitis and endocarditis, in pigs. We are currently working on the construction of a biofilm-deficient mutant of strain 95-8242 that could be used in an experimental animal model to investigate the role of biofilm formation in pathogenicity. Since biofilm-grown bacteria are more resistant to antibiotics, longer periods of antibiotic therapy, higher doses of antibiotics or a better selection of antibiotics may be required to resolve infections caused by strains having the capacity to produce biofilms.

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