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ORIGINAL ARTICLE

Production of cell–cell signalling molecules by bacteria isolated from human chronic wounds

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Abstract

Aim: To (i) identify chronic wound bacteria and to test their ability to produce acyl-homoserine-lactones (AHLs) and autoinducer-2 (AI-2) cell–cell signalling molecules and (ii) determine whether chronic wound debridement samples might contain these molecules.

Methods and Results: Partial 16S rRNA gene sequencing revealed the identity of 46 chronic wound strains belonging to nine genera. Using bio-reporter assays, 69·6% of the chronic wound strains were inferred to produce AI-2, while 19·6% were inferred to produce AHL molecules. At least one strain from every genus, except those belonging to the genera *Acinetobacter* and *Pseudomonas*, were indicated to produce AI-2. Production of AI-2 in batch cultures was growth-phase dependent. Cross-feeding assays demonstrated that AHLs were produced by *Acinetobacter* spp., *Pseudomonas aeruginosa* and *Serratia marcescens*. Independent from studies of the bacterial species isolated from wounds, AHL and/or AI-2 signalling molecules were detected in 21 of 30 debridement samples of unknown microbial composition.

Conclusion: Chronic wound bacteria produce cell–cell signalling molecules. Based on our findings, we hypothesize that resident species generally produce AI-2 molecules, and aggressive transient species associated with chronic wounds typically produce AHLs. Both these classes of cell–cell signals are indicated to be present in human chronic wounds.

Significance and Impact of the Study: Interbacterial cell–cell signalling may be an important factor influencing wound development and if this is the case, the presence of AHLs and AI-2 could be used as a predictor of wound severity. Manipulation of cell–cell signalling may provide a novel strategy for improving wound healing.

Introduction

Chronic wounds currently affect 2·5–4·5 million people in the US (Jones *et al.* 2007). This is a major health problem as chronic wounds are unresponsive to typical treatment strategies and persist even after the implementation of normal wound debridement, wound irrigation and antimicrobial-based treatment strategies (Dow *et al.* 1999; Harding *et al.* 2002). Three common types of chronic wounds are described by their aetiology and include those related to neuropathic disorders (e.g. diabetic ulcers and diabetic foot ulcers or DUs), vascular disorders (e.g. venous leg ulcers or VLUs) and environmentally induced disorders [e.g. pressure ulcers or PUs, nonhealing surgical wounds or NHSWs and general chronic nonhealing wounds (NHWs)] (Mustoe *et al.* 2006; Wolcott *et al.* 2008). While their aetiology is distinct, a commonality is the presence of high concentrations of bacteria within the wounds (Wolcott *et al.* 2008).

Recently, James *et al.* (2008) demonstrated that many species of bacteria exist within chronic wounds as part of complex multispecies biofilms. This is a significant finding as bacteria within biofilms exhibit properties that are distinct from planktonic cells (Donlan and Costerton 2002; Fux *et al.* 2005; Spormann 2008). In particular, biofilm bacteria are more resistant to antimicrobials than their planktonic counterparts (Gilbert *et al.* 2002) and are also resilient to attack from the human immune system (Donlan and Costerton 2002). In these resilient chronic wound biofilms, two distinct bacterial populations exist. One population can be described as the resident bacterial population, which multiplies and persists on the skin of healthy individuals, and the other is the transient bacterial population, which colonizes and multiplies in unhealthy human skin and is typically found in high concentrations in chronic wound biofilms (Price 1938; Ruocco *et al.* 2007). Biofilm community analysis of chronic wounds have shown not only the presence of typically resident species (often referred to as commensals) of the normal cutaneous microbial flora, such as *Streptococcus* spp., *Proteus mirabilis*, *Bacillus* spp. and *Staphylocccus* spp. (Dekio *et al.* 2005; Gjodsbol *et al.* 2006; Dowd *et al.* 2008a; James *et al.* 2008), but also the presence of high cell numbers transient species (that may have the potential to be skin pathogens) which are typically Gram-negative rod-shaped bacteria (Lowbury 1969) such as *Pseudomonas aeruginosa* (Gjodsbol *et al.* 2006) and *Acinetobacter* spp. (Bowler and Davies 1999; Hill *et al.* 2003). These transient species are considered to be highly aggressive chronic wound biofilm species as they are able divide rapidly to numerically dominate within chronic wound multispecies biofilms. Indeed, a recent study by Gjodsbol *et al.* (2006) demonstrated that chronic wounds that harboured *Ps. aeruginosa* within the biofilms were significantly larger than those without *Ps. aeruginosa*. An ability to control the development of multispecies biofilms and specifically inhibit pathogenic species within a biofilm would be beneficial in the treatment of chronic wounds.

The production and detection of bacterial cell–cell signalling molecules by species have been repeatedly linked to the enhanced development of single and multispecies biofilms (Irie and Parsek 2008). A variety of structurally different bacterial cell–cell signalling molecules have been shown to mediate cell–cell communication and include acylated homoserine lactones (AHLs) and autoinducer-2 (AI-2) molecules. AHLs are produced solely by Gram-negative bacteria (Williams 2007), although different species often produce one or more different forms of AHLs. These different AHLs all consist of a homoserine lactone (HSL) ring moiety but differ with respect to the length, degree of saturation and specific substitutions within an attached acyl side-chain. Because of the heterogeneity of AHL structure(s), AHLs have been proposed to mediate intraspecies bacterial communication; different species typically only recognize AHLs produced from closely related species (Miller and Bassler 2001). However, there are examples where bacterial species can detect AHLs from bacteria belonging to distantly related genera (Stickler *et al.* 1998; Bernier *et al.* 2008). Conversely, another class of cell–cell signalling molecule, called AI-2, has been shown to mediate interspecies signalling (Surette *et al.* 1999; Yoshida *et al.* 2005; Rickard *et al.* 2006). AI-2 is an umbrella term used to describe a family of interconvertible molecules that is derived from the same precursor molecule; 4,5-dihydroxy-2,3-pentanedione (DPD) (Semmelhack *et al.* 2005). DPD is produced or detected by many Gram-positive and Gram-negative bacteria (Sun *et al.* 2004) and has been shown to contribute to single-species and dual-species biofilm development (Gonzalez Barrios *et al.* 2006; Rickard *et al.* 2006; Hardie and Heurlier 2008).

Recently, work by Nakagami *et al.* (2008) has shown that AHLs can be detected in pressure-induced infected ischemic wounds on rats. Of particular interest, was the finding that wounds that were infected with *Ps. aeruginosa* contained AHLs at concentrations up to 0·49 pmol g^{-1}. The amount of AHLs increased linearly as the cell density of *Ps. aeruginosa* in the wound also increased. No AHLs were detected in wounds that were not infected with *Ps. aeruginosa.* The amount of other cell signalling molecules, such as AI-2, was not determined.

To increase understanding of the role of bacterial cell–cell signalling in the development of human chronic wounds, it was the aim of this study to determine whether bacteria that are often isolated from typical chronic wound biofilms produce cell–cell signalling molecules. It was also the aim of this work to determine whether these signal molecules could be detected in aetiologically distinct wounds. We discovered that many different species of chronic wound bacteria produce cell–cell signalling molecules and most resident species produce AI-2, while Gram-negative pathogens produce only AHLs. A study of chronic wound debridement samples of unknown microbial composition, but from different wound types (DUs, VLUs, NHWs and NHSWs), also indicates the presence of possible AHLs or AI-2 molecules in chronic wounds.

Materials and methods

Strains and growth conditions

Forty-six strains were isolated from chronic wound debridement specimens from 32 patients undergoing

standard sharp debridements as part of the normal course of their wound care management. These specimens were collected with written informed consent under a protocol approved by the Western Institutional Review Board (Olympia, WA, USA). Strains were randomly chosen from culturable bacterial panels generated from debridement samples taken from patients suffering with DUs, PUs, NHSWs, NHWs or VLUs. The source of each strain is shown in Table 1. Strains were cultured in Schaedler broth (BD, Franklin Lakes, NJ,

Table 1 Strains isolated from chronic wound debridement samples. Assigned EMBL accession numbers are included

Wound type	Strain	Most closely related species or genus	Sequence length (bp)	Identity (%)	Assigned identity	EMBL accession number
NHW	CWS1	*Staphylococcus aureus*	753	98	*Staph. aureus*	FM207477
NHW	CWS34	*Staph. aureus*	750	99	*Staph. aureus*	FM207534
PU	CWS30	*Staph. aureus*	748	98	*Staph. aureus*	FM207530
PU	CWS31	*Staph. aureus*	758	97	*Staphylococcus* sp.	FM207531
PU	CWS41	*Staph. aureus*	765	99	*Staph. aureus*	FM207541
VLU	CWS37	*Staph. aureus*	751	100	*Staph. aureus*	FM207537
DU	CWS5	*Serratia marcescens*	755	98	*Ser. marcescens*	FM207481
DU	CWS35	*Ser. marcescens*	759	98	*Ser. marcescens*	FM207535
NHW	CWS39	*Ser. marcescens*	751	99	*Ser. marcescens*	FM207539
NHW	CWS44	*Ser. marcescens*	753	99	*Ser. marcescens*	FM207544
PU	CWS40	*Ser. marcescens*	741	99	*Ser. marcescens*	FM207540
VLU	CWS4	*Ser. marcescens*	752	99	*Ser. marcescens*	FM207480
VLU	CWS25	*Ser. marcescens*	751	100	*Ser. marcescens*	FM207962
DU	CWS7	*Pseudomonas aeruginosa*	750	99	*Ps. aeruginosa*	FM207483
DU	CWS12	*Ps. aeruginosa*	698	99	*Ps. aeruginosa*	FM207514
DU	CWS13	*Ps. aeruginosa*	747	99	*Ps. aeruginosa*	FM207515
DU	CWS14	*Ps. aeruginosa*	712	99	*Ps. aeruginosa*	FM207516
PU	CWS29	*Ps. aeruginosa*	742	98	*Ps. aeruginosa*	FM207529
PU	CWS33	*Ps. aeruginosa*	710	98	*Ps. aeruginosa*	FM207533
VLU	CWS3	*Ps. aeruginosa*	748	99	*Ps. aeruginosa*	FM207479
NHW	CWS22	*Proteus mirabilis*	748	99	*Pr. mirabilis*	FM207524
NHW	CWS27	*Pr. mirabilis*	748	99	*Pr. mirabilis*	FM207527
PU	CWS23	*Pr. mirabilis*	738	99	*Pr. mirabilis*	FM207525
PU	CWS48	*Pr. mirabilis*	791	100	*Pr. mirabilis*	FM207476
VLU	CWS11	*Pr. mirabilis*	750	99	*Pr. mirabilis*	FM207513
VLU	CWS26	*Pr. mirabilis*	744	99	*Pr. mirabilis*	FM207526
NHSW	CWS38	*Klebsiella pneumoniae*	742	100	*Kl. pneumoniae*	FM207538
NHW	CWS45	*Kl. pneumoniae*	779	99	*Kl. pneumoniae*	FM207545
NHW	CWS47	*Kl. pneumoniae*	713	96	*Klebsiella* sp.	FM207963
VLU	CWS42	*Klebsiella oxytoca*	748	98	*Kl. oxytoca*	FM207542
VLU	CWS19	*Escherichia coli*	743	98	*E. coli*	FM207521
VLU	CWS32	*E. coli*	749	100	*E. coli*	FM207532
DU	CWS6	*Enterococcus faecalis*	766	99	*Ent. faecalis*	FM207482
DU	CWS15	*Ent. faecalis*	702	98	*Ent. faecalis*	FM207517
DU	CWS17	*Ent. faecalis*	743	100	*Ent. faecalis*	FM207519
NHSW	CWS36	*Ent. faecalis*	772	100	*Ent. faecalis*	FM207519
NHW	CWS46	*Ent. faecalis*	769	99	*Ent. faecalis*	FM207546
PU	CWS8	*Ent. faecalis*	736	99	*Ent. faecalis*	FM207484
PU	CWS16	*Ent. faecalis*	730	98	*Ent. faecalis*	FM207518
VLU	CWS10	*Ent. faecalis*	743	99	*Ent. faecalis*	FM207512
VLU	CWS21	*Ent. faecalis*	754	99	*Ent. faecalis*	FM207523
NHSW	CWS43	*Bacillus mojavensis*	757	99	*B. mojavensis*	FM207543
NHW	CWS28	*Acinetobacter lwoffii*	733	99	*Ac. lwoffii*	FM207528
VLU	CWS9	*Acinetobacter calcoaceticus*	730	98	*Ac. calcoaceticus*	FM207485
VLU	CWS20	*Ac. calcoaceticus*	737	99	*Ac. calcoaceticus*	FM207522
VLU	CWS18	*Acinetobacter baumannii*	735	99	*Ac. baumannii*	FM207520

DU, diabetic ulcer; VLU, venous leg ulcer; PU, pressure ulcer; NHSW, nonhealing surgical wound; NHW, nonhealing wound.

USA) supplemented with 1 mmol l^{-1} boric acid. Liquid cultures were grown aerobically at 37°C with shaking at 200 rev min^{-1}.

Vibrio harveyi strains BB170, BB152, BB886 and BB120 (Surette and Bassler 1998) were used to detect AI-2 (DPD) and the AHL *N*-(3-hydroxybutanoyl) homoserine lactone (HBHL). *Vibrio harveyi* BB170 detects AI-2 while *V. harveyi* BB152 is a producer of AI-2 and was used as a positive control. *Vibrio harveyi* BB886 detects HBHL (also referred to as autoinducer-1) while *V. harveyi* BB120 is a producer of HBHL and was used as a positive control. All *V. harveyi* strains were cultured in autoinducer bioassay (AB) medium (Greenberg *et al.* 1979) at 30°C. Liquid cultures were grown aerobically at 30°C with shaking at 200 rev min^{-1}.

Agrobacterium tumefaciens strains were used to detect other forms of AHL which include *N*-3-(oxo-octanoyl) homoserine lactone (referred to as OOHL) as well as a wide range of structurally similar AHLs (Stickler *et al.* 1998). *Agrobacterium tumefaciens* KYC6 (Stickler *et al.* 1998), a producer of OOHL and other AHL molecules, was grown on LB agar at 30°C. *Agrobacterium tumefaciens* A136 (Stickler *et al.* 1998), which detects OOHL and other AHLs and responds by producing β-galactosidase, was grown in LB supplemented with spectinomycin (50 μg ml^{-1}) and tetracycline (4·5 g m^{-1}) at 30°C. All strains were stored at −70°C in 50% glycerol.

Preparation of crude cell-free extract from chronic wound samples

Separate to the collection of samples for the isolation of bacterial strains for this study, 30 human debridement samples were collected and frozen at −70°C until extraction of cell-free supernatant was required. Prior to extraction, the weight of each thawed sample was recorded and 500 μl of de-ionized water was added to the debridement sample. Samples were shaken vigorously using a Qiagen TissueLyser (Qiagen, Valencia, CA, USA) at 30 Hz for 2×30 s. Subsequently, samples were centrifuged at 13 000 ***g*** for 120 s. The supernatant was collected and filtered through a 0·45 μm Millex® HA filter unit (Millipore, Bedford, MA, USA). Cell-free debridement samples were frozen immediately at −70°C for future use.

Cross-signalling assay for the detection of a broad range of AHL molecules

To determine the production of a broad range of AHLs from chronic wound strains, the AHL detector strain *Ag. tumefaciens* A136 was used in a cross-feeding assay developed by Stickler *et al.* (1998). LB agar was prepared by covering with 100 μl of 20 mg ml^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). *Agrobacterium tumefaciens* A136 was streaked onto the prepared LB agar and chronic wound strains were streaked in parallel *c.* 1 cm away. Plates were then incubated at 30°C for 48 h. If OOHL, or one of a variety of other AHLs with different acyl side-chain lengths and substitutions (6–12 carbon atoms in length), were produced by a test strain, then *Ag. tumefaciens* A136 reported the presence of AHL molecules by activating a *traI–lacZ* fusion. Activation of the reporter fusion results in the cleavage of X-Gal which turns *Ag. tumefaciens* A136 colonies blue. Positive and negative controls consisted of culturing the reporter strain with the AHL over-producer strain *Ag. tumefaciens* KYC6 and with itself (an AHL nonproducer).

To determine the presence of a broad range of AHLs in chronic wounds, the AHL detector strain *Ag. tumefaciens* A136 was also used. To detect AHL cell–cell signalling molecules, 6·5- mm diameter discs of Whatman filter paper (Number One; Whatman, Hillsboro, OR, USA) were impregnated with 5 μl of cell-free supernatant from chronic wounds. These discs were placed onto the surface of LB agar plates supplemented with 50 μg ml^{-1} spectinomycin and 4·5 μg ml^{-1} tetracycline and precoated with 100 μl of X-Gal (20-mg ml^{-1} stock solution in dimethyl formamide) and 100 μl of a cell suspension (OD 1·0 at 610 nm) of *Ag. tumefaciens* A136. Plates were incubated for 72 h at 30°C, and 5 μl from cell-free chronic wound supernatant was added to each respective disc every 12 h. The presence of AHLs in wound samples was inferred if the *Ag. tumefaciens* A136 colonies in contact with the disc or surrounding the disc became blue after 72 h incubation.

Bioluminescence assays for the detection of AI-2 and *Vibrio harveyi* AHL (HBHL) molecules

HBHL (an AHL that is also referred to as autoinducer-1) and AI-2 activity were determined by testing batch culture cell-free supernatants using a modification of a bioluminescence assay by Frias *et al.* (2001). Cells of *V. harveyi* BB170 (AI-2 reporter strain) or BB886 (HBHL reporter strain) were grown at 30°C in batch cultures of AB medium according to the method of Surette and Bassler (1998). After 14 h of growth, *V. harveyi* BB170 or *V. harveyi* BB886 cultures were diluted 1 : 500 in fresh AB medium (Rickard *et al.* 2006). The diluted *V. harveyi* BB170 or *V. harveyi* BB886 cultures were stored at −70°C until required.

To analyse the amount of HBHL or AI-2 produced by chronic wound strains, each strain was grown individually in batch cultures containing Schaedler medium supplemented with 1 mmol l^{-1} boric acid. Following 3, 6·5, 8·5 and 24 h batch-culture growth, 1 ml of cultures was collected and passed through a 0·2- μm filter unit (Fisher Scientific, Suwanee, GA, USA). From these cell-

Table 2 Induction of cell–cell signal reporter strains by chronic wound strains. *Agrobacterium tumefaciens* A136 detects *N*-3-(oxo-octanoyl) homoserine lactone, or one of a variety of other acyl-homo-serine-lactones (AHLs) (6–12 carbon atoms in length), *Vibrio harveyi* BB886 detects the presence of *N*-(3-hydroxybutanoyl) homoserine lactone (HBHL) and *V. harveyi* BB170 detects the presence of autoinducer-2 (AI-2). Positive results are highlighted in bold

Strain	*Ag. tumefaciens* A136	*V. harveyi* BB886*	*V. harveyi* BB170
V. harveyi BB152†	ND	ND	631·6
V. harveyi BB120‡	ND	2776·2	ND
Escherichia coli DH5α§	–	0·4	12·8
Staphylococcus aureus CWS1	–	1·4	**33·2**
Staph. aureus CWS34	–	0·3	**137**
Staph. aureus CWS30	–	0·3	**164·9**
Staphylococcus sp. CWS31	–	0·3	23·3
Staph. aureus CWS41	–	0·3	**75·6**
Staph. aureus CWS37	–	0·2	24·7
Serratia marcescens CWS5	+	1·8	**271·6**
Ser. marcescens CWS35	–	1·3	**30·1**
Ser. marcescens CWS39	–	0·1	**30·4**
Ser. marcescens CWS44	+	9·6	**134·5**
Ser. marcescens CWS40	–	3	**35·4**
Ser. marcescens CWS4	+	2·2	**205·3**
Ser. marcescens CWS25	–	2·3	**45·5**
Pseudomonas aeruginosa CWS7	–	1	4·3
Ps. aeruginosa CWS12	+	0·5	13·7
Ps. aeruginosa CWS13	+	2·8	2·16
Ps. aeruginosa CWS14	+	0·3	11
Ps. aeruginosa CWS29	–	0·5	0·57
Ps. aeruginosa CWS33	+	0	1·02
Ps. aeruginosa CWS3	–	0·2	10·3
Proteus mirabilis CWS22	–	0·8	**110**
Pr. mirabilis CWS27	–	2·6	**69·4**
Pr. mirabilis CWS23	–	1·5	**133**
Pr. mirabilis CWS48	–	0·2	**44·4**
Pr. mirabilis CWS11	–	2·6	**382·5**
Pr. mirabilis CWS26	–	1·9	**63·3**
Klebsiella pneumoniae CWS38	–	0·1	**30·7**
Kl. pneumoniae CWS45	–	1·1	**168·3**
Klebsiella sp. CWS47	–	0·7	**174**
Klebsiella oxytoca CWS42	–	0·2	**44·4**
E. coli CWS19	–	1·3	**196·1**
E. coli CWS32	–	8·8	**99·3**
Enterococcus faecalis CWS6	–	0·5	**165·7**
Ent. faecalis CWS15	–	1·6	**29·8**
Ent. faecalis CWS17	–	2·0	**182·3**
Ent. faecalis CWS36	–	5·2	**295·6**
Ent. faecalis CWS46	–	0·6	**201·5**
Ent. faecalis CWS8	–	1·1	**191·5**
Ent. faecalis CWS16	–	0·2	**30·8**
Ent. faecalis CWS10	–	0·7	**89·3**
Ent. faecalis CWS21	–	2	19·82
Bacillus mojavensis CWS43	–	1·2	**40·74**
Acinetobacter lwoffii CWS28	–	0·8	0·75
Acinetobacter calcoaceticus CWS9	–	1·9	3·4
Ac. calcoaceticus CWS20	+	0·7	1·7
Acinetobacter baumannii CWS18	+	1·2	4·2

**Vibrio harveyi* BB886 reporter strain did not bioluminesce strongly upon exposure to cell-free culture media from the chronic wound strains (≤9·6-fold induction) and this indicates that none produced HBHL (AI-1).

†*V. harveyi* BB152 is a positive control and produces AI-2.

‡*V. harveyi* BB120 is a positive control and produces AHL.

§*Escherichia coli* DH5α is a negative control and does not produce AI-1 or AI-2.

free supernatants, 10 μl was added to 90 μl of thawed diluted *V. harveyi* BB170 culture (to detect AI-2) or to 90 μl of thawed diluted *V. harveyi* BB886 culture (to detect *V. harveyi* HBHL) in a 96-well microplate and analysed in a Victor 3 multilabel counter (Perkin Elmer, Waltham, MA, USA). Bioluminescence values that were relative to un-inoculated medium were calculated as fold inductions (Blehert *et al.* 2003). For batch cultures studies, a positive result was recorded for fold inductions of ≥30 over the media control. A 30-fold induction represents a fold induction that is 1% of the *V. harveyi* HBHL positive control (*V. harveyi* BB120) and 5% of the *V. harveyi* AI-2 positive control (*V. harveyi* BB152) (Table 2). The higher 5% cut-off point for determining if strains produce AI-2 was chosen because the addition of 1 mmol l^{-1} boric acid to growth media not only strengthens the signal from AI-2-producing strains but also enhances background signal (DeKeersmaecker and Vanderleyden 2003). A comparison to *Escherichia coli* DH5α, that is known to be unable to produce AI-2 in boric acid-supplemented Schaedler medium, shows that 5% was an appropriate cut-off (Table 2).

To determine the presence of HBHL or AI-2 in chronic wounds, *V. harveyi* BB886 (HBHL detector) and *V. harveyi* BB170 (AI-2 detector) were used. To detect the presence of HBHL or AI-2 molecules, 10 μl of wound sample was added to 90 μl of either thawed diluted *V. harveyi* BB170 culture or *V. harveyi* BB886 culture in a 96-well microtitre plate and analysed in a Victor 3 Victor 3 multilabel counter. Bioluminescence relative to PBS (pH 7·4) was calculated as fold inductions (Blehert *et al.* 2003). Because wound samples were not supplemented with boric acid, fold inductions >10 (1% of typical bioluminescence from cell-free supernatants of *V. harveyi* BB152) were considered positive for AI-2 signal production. Samples that generated a fold induction of >20

bioluminesence from *V. harveyi* BB886 were also considered positive for HBHL.

Identification of strains by 16S rRNA gene sequencing

Following a modified method of Rickard *et al.* (2002), strains were identified by polymerase chain reaction (PCR) amplification and partial sequencing of the 16S rRNA gene fragment. Amplification of 16S rRNA was performed by taking one 72- h-old colony of each strain grown on Schaedler agar. The colonies were boiled separately in 100 μl of double-autoclaved nanopure water for 7 min, and 10 μl of each boiled suspension was used as template DNA for PCR amplifications. Degenerate primers 8FPL (Wilson *et al.* 1990) and 806R (Weisburg *et al.* 1991) were used to amplify fragments of 16S rDNA which correspond to nucleotides 8–806 in the *E. coli* K12 16S rRNA gene sequence. PCR were carried out in PCR Red-Taq reaction buffer (Sigma, St Louis, MO, USA). PCR consisted of 35 cycles at 94°C (1 min), 53·5°C (1 min) and 72°C (1 min), plus a final cycle with a 15 min chain elongation step at 72°C. Amplified products were purified (QIA-quick™ PCR purification kit; Qiagen, Warrington, UK), and purified PCR products were sequenced using the primers 806R and 8FPL. Sequencing reactions consisted of 20–80 ng of PCR product, 10 ng of primer and 2 μl of Big Dye™ (PE Applied Biosystems, Foster City, CA, USA) in a total volume of 20 μl. Samples were incubated at 94°C (4 min) followed by 25 cycles of 96°C (30 s), 50°C (15 s) and 60°C (4 min). Sequencing was performed in a Perkin-Elmer ABI 310 sequencer (Perkin-Elmer, Foster City, CA). Unambiguous sequences of 650–750 bases in length were obtained from each strain and compared to known sequences in the EMBL database using Blast (Altschul *et al.* 1997). Based on the criteria described by Stackebrandt and Goebel (1994), 16S rRNA gene sequences that were 97–100% identical to speciated strains in the EMBL database were assigned the genus and species name. Sequences that possessed a sequence identity of <97% to speciated strains in the EMBL database were only assigned the genus name.

Tree construction

The closest relative species or genera were assigned based upon compiled partial 16S rRNA gene sequences. Representative sequences of closely related strains were also aligned using ClustalX ver. 1.83 (Thompson *et al.* 1997). Neighbour-joining analysis was conducted with the correction of Jukes and Cantor (1969) using Treecon ver. 1.3b (Van de Peer and De Wachter 1993) with *Thermus thermophilus* (X07998) as the out-group.

Results

Identification and phylogenetic relatedness of strains

All strains were identified to the genus level and, based upon the criteria of Stackebrandt and Goebel (1994), all but two strains (*Klebsiella* sp. CWS47 and *Staphylococcus* sp. CWS31) were speciated (Table 1). The strains belonged to nine genera and 33 of 46 strains shared ≥99% identity to known 16S rRNA gene sequences from previously identified strains that are commonly isolated from chronic wounds. These include *Serratia marcescens*, *E. coli*, *Enterococcus faecalis*, *Ps. aeruginosa* and *Staphylococcus aureus*. The un-speciated strains, *Klebsiella* sp. CWS47 and *Staphylococcus* sp. CWS31, were not given species epithets as they shared an identity of ≤97% to the nearest speciated 16S rRNA gene sequence in the EMBL database.

Partial 16S rRNA gene sequence alignment, nearest-neighbour pair-wise comparison and construction of a phylogram (Fig. 1) confirmed that the assigned identity of each chronic wound strain was appropriate. Additionally, the tree revealed that some strains of the same genera shared identical partial 16S rRNA gene sequences and some were less similar. For example, the partial 16S rRNA from *Ent. faecalis* CWS36 was identical to *Ent. faecalis* CWS17, while *Ent. faecalis* CWS15 was <98% identical to both *Ent. faecalis* CWS36 and *Ent. faecalis* CWS17. None of the strains with identical 16S rRNA gene sequences were isolated from the same individual (data not presented). Similar patterns of sequence identities were observed for clusters of strains belonging to *Pr. mirabilis*, *Ser. marcescens*, *Ps. aeruginosa* and *Staph. aureus*. Different patients with different wounds could harbour closely related or less-related strains of the same genera.

Production of AHL molecules by chronic wound strains

Two approaches were used to determine if the chronic wound bacteria produce AHLs. Both approaches relied upon the growth of the organisms in laboratory media. The first approach used a bioluminescence assay, developed using *V. harveyi* BB886 to detect HBHL (Surette and Bassler 1998). The second approach used a cross-feeding assay, developed by Stickler *et al.* (1998) and relies upon the reporter strain *Ag. tumefaciens* A136 to detect the presence of a broad range of different forms of AHL molecules (homoserine lactone acyl side-chain lengths of C6–C12).

According to the *V. harveyi* BB886 bioluminescence assay, none of the chronic wound bacteria induced bioluminescence that was consistently >30·0-fold over the negative control and only generated signals that were

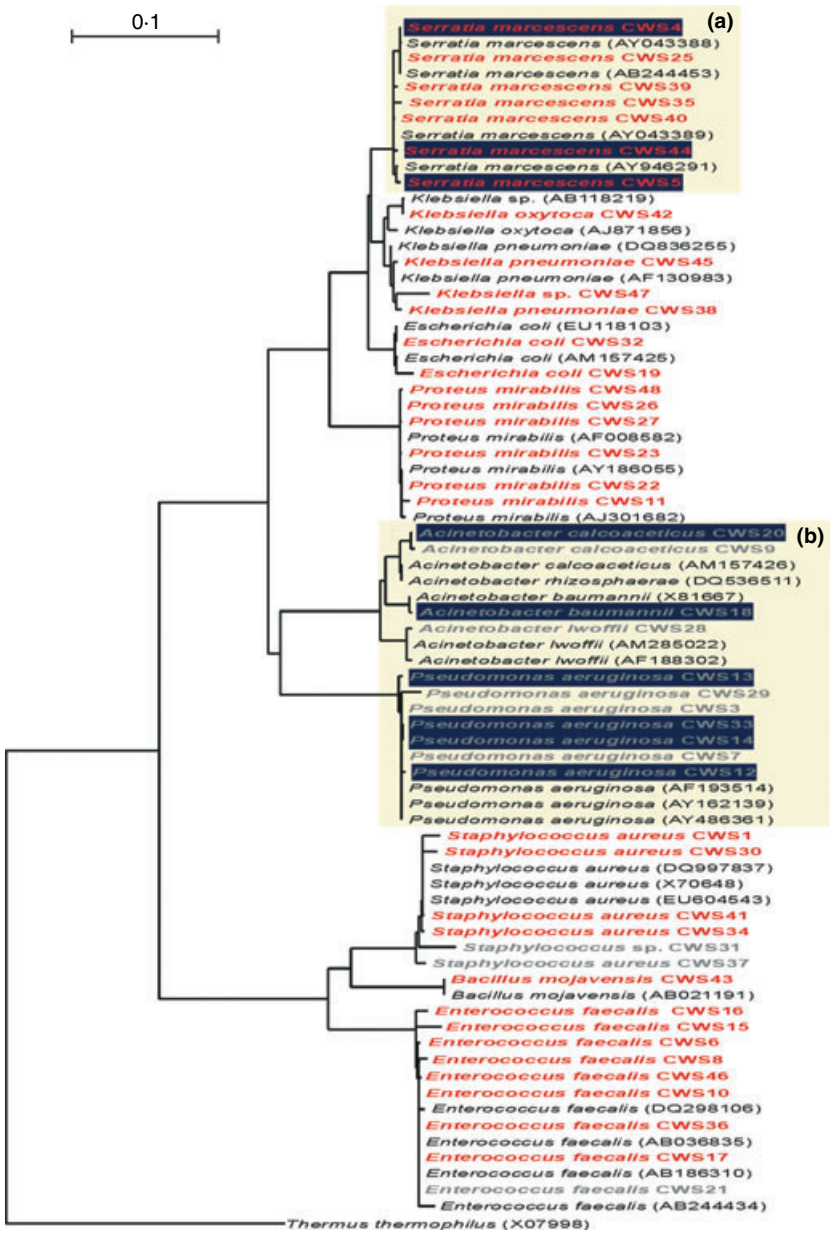

Figure 1 Neighbour-joining phylogenetic tree constructed using partial 16S rRNA gene sequences of chronic wound isolates and closely related published speciated strains. The tree is rooted using the out-group sequence from *Thermus thermophilus* (X07998). Nucleotide sequence database accession codes are shown in the brackets. The length of each branch is proportional to the estimated number of substitutions per position. Bar represents one nucleotide substitution for every ten nucleotides. Sequenced chronic wound strains are labelled with the identifier CWS followed by a strain number and most likely identities are assigned. Colours represent the inferred production of acyl-homoserine-lactone (AHL) or autoinducer-2 (AI-2) cell–cell signal molecules by that strain. Strain names labelled with red text are those that produce AI-2 (using the reporter strain *Vibrio harveyi* BB170), while blue boxes label those strains that produce AHL molecules (using the reporter strain *Agrobacterium tumefaciens* A136). Text of strains that are only coloured grey represents strains that did not produce detectable quantities of either signal molecule. None of the strains produced *N*-(3-hydroxybutanoyl) homoserine lactone molecules that were detectable by *V. harveyi* BB886. Yellow boxes highlight those genera that (a) produce AHL and AI-2 and (b) those that produce only AHL.

≤0·4% of that from the positive control, *V. harveyi* BB120 (Table 2). Thus, all the chronic wound strains were deemed not to produce HBHL signal molecules. Cross-feeding assays, using *Ag. tumefaciens* A136, demonstrated that 19·6% of the strains (9 of 46) produced AHL molecules (other than HBHL) (Table 2).

Only 50% (9 of 18) of the strains belonging to *Ser. marcescens*, *Ps. aeruginosa* and *Acinetobacter* spp. were inferred to produce AHL signal molecules (Fig. 1a,b). The reason why AHL cell–cell signal molecules were not detected for all strains belonging to these genera may be because of the absence of detectable quantities of AHL being produced by the seemingly non-AHL-producing strains. A negative response does not necessarily mean that these AHL-negative strains do not produce AHL, and instead may mean the amount produced is below the threshold for detection in this reporter system. *Acinetobacter baumannii* CWS18 elicited the strongest reaction from the *Ag. tumefaciens* A136 reporter strain. Such a reaction, inferred by the induction of β-galactosidase production and development of a blue colony type, may indicate that this strain produced a collection of different detectable AHL signal molecules or one type of AHL signal molecule at high concentration.

Production of AI-2 molecules by chronic wound strains

With the exception of strains belonging to the genera *Pseudomonas* and *Acinetobacter*, at least one strain belonging to each of the other seven genera was inferred to produce AI-2 (Fig. 1, Table 2). The three highest AI-2 fold-induction values were obtained from batch cultures of *Pr. mirabilis* CWS11 (382·5-fold induction), *Ent. faecalis* CWS36 (295·6-fold induction) and *Ser. marcescens* CWS5 (271·6-fold induction).

The amount of AI-2 detected, from AI-2-producing strains, varied during batch-culture growth (four sample points in different growth phases in batch culture), and changes in amounts of AI-2 was different for each species. For example, differences in AI-2 concentration (expressed as fold induction) occurred over different time points and growth phases in batch cultures of *Ent. faecalis* CWS8 and in batch cultures of *Staph. aureus* CWS41 (Fig. 2). *Staphylococcus aureus* CWS41 produced AI-2 in exponential phase and, upon entry into stationary phase, either halted production of AI-2 or sequestered (through cellular-uptake) AI-2 at the same rate at which it was being produced. Conversely, *Ent. faecalis* CWS8 produced AI-2 in exponential phase and early stationary phase, but the amount of AI-2 that was present in later stationary phase cultures was reduced by 74%, indicating that AI-2 was being internalized or degraded extracellulary.

Detection of cell–cell signal molecules in chronic wound debridement samples

Thirty chronic wound debridement samples were analysed to determine if AHL and/or AI-2 molecules could be detected. Samples were taken from four aetiologically

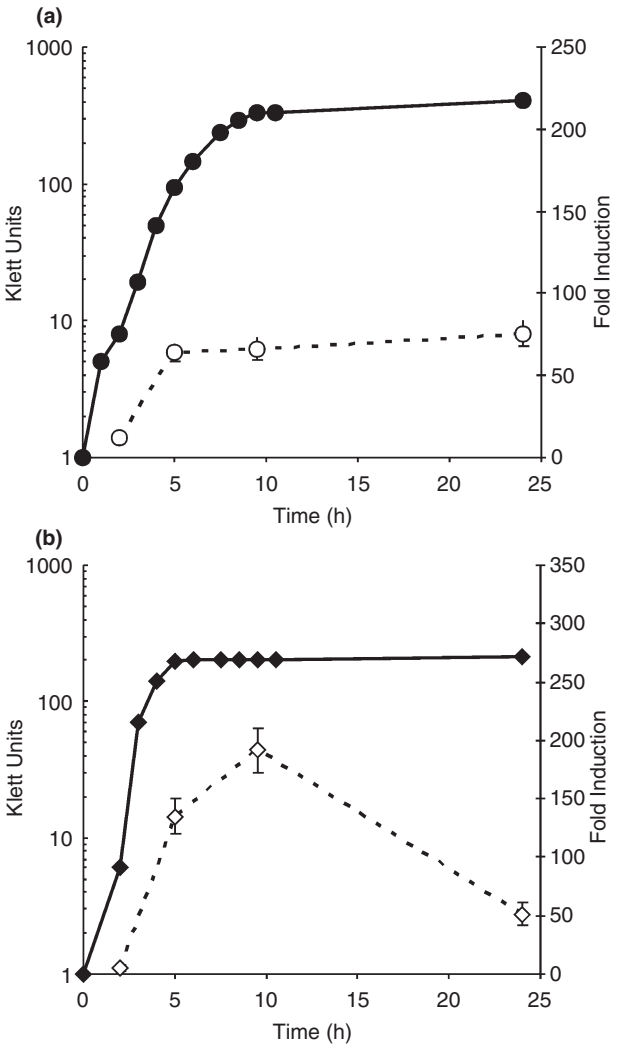

Figure 2 Kinetics of growth and autoinducer-2 (AI-2) production for (a) *Staphylococcus aureus* CWS41 (b) *Enterococcus faecalis* CWS8. Solid symbols denote the growth kinetics and open symbols denote the fold induction of bioluminescence over a media control. Fold induction is the increase in light emission by *Vibrio harveyi* BB170, when exposed to cell-free supernatants of strains grown in batch culture over the level of luminescence obtained from the corresponding sterile growth medium. Fold induction is an indicator of the amount of AI-2 in the batch culture. The data are reported as *V. harveyi* BB152 supernatant generated an average of a 631·6-fold induction.

different types of chronic wounds (Table 3). The *V. harveyi* BB886 bioluminescence assay, which detects HBHL, showed that none of the samples likely contained detectable quantities of HBHL as bioluminescence was <9·42-fold induction over bioluminescence induced by phosphate-buffered saline (Table 3). Use of *Ag. tumefaciens* A136 as a bio-reporter for other AHL molecules indicated that 15 of 30 of the samples contained detectable concentrations of AHL molecules other than HBHL

Table 3 Induction of cell–cell signal reporter strains by cell-free fluids from chronic wound debridement samples. Samples were taken from diabetic ulcers (DUs), nonhealing surgical wounds (NHSWs), nonhealing wounds (NHWs) or venous leg ulcers (VLUs). Bold symbols or values are considered positive results

Wound sample	Sample weight (mg)	*Agrobacterium tumefaciens* A136	*Vibrio harveyi* BB886	*V. harveyi* BB170
DU	499	**+**	0·5	1·1
DU	1309	**+**	0·4	0·3
DU	175·8	–	1·3	**55·1**
DU	463·9	–	0·8	0·3
DU	199·2	**+**	0·8	0·4
DU	352·9	–	1·2	0·2
DU	215·4	**+**	1·5	0·2
DU	47·5	–	9·4	**32·4**
DU	74·5	–	3·7	**156·1**
DU	95	**+**	2	**36·2**
DU	185·7	**+**	5·4	**25·5**
DU	326·3	**+**	1·5	0·5
DU	231	**+**	6·7	0·3
NHSW	298	–	1·9	**185·5**
NHSW	90·7	**+**	3·1	0·7
NHSW	205·6	**+**	3·3	0·3
NHSW	333·5	**+**	2·2	0·2
NHW	282	**+**	0·3	1·9
NHW	498	–	0·5	3·1
NHW	1135	–	0·5	0·5
VLU	203	–	1	**61·5**
VLU	438	–	0·6	0·1
VLU	505	**+**	0·5	0·3
VLU	751	–	0·3	2·1
VLU	363·9	**+**	1·4	0·5
VLU	258·3	–	0·9	**14·3**
VLU	49·9	–	5·9	1·2
VLU	389·7	–	2·5	5·5
VLU	96·7	**+**	1·4	1·6
VLU	80·5	–	2·1	2·9

(Table 3). The presence of these AHL molecules was inferred by a change in the colour (from off-white to blue) of *Ag. tumefaciens* A136 colonies in contact and around cell-free wound debridement sample impregnated discs. In all positive cases, colonies of *Ag. tumefaciens* A136 growing on or in close proximity to the discs impregnated with chronic wound debridement sample turned a shade of blue and colonies that were further away from the discs were a often lighter blue or remained white (Fig. 3). Of interest too, was that for some wound samples, a zone of inhibition was evident within 1–5 mm around the disc and was probably because of the presence of human-cell-produced inhibitory components or antibiotics from treatment regimes. Of the entire wound debridement samples tested, those from DUs most often inferred to contain AHLs (61·5%, Table 3). Debridement samples from NHSWs and NHWs and VLUs least often contained AHLs (57·1% and 30%, respectively, Table 3).

Analysis of the cell-free extracts of debridement samples, using the *V. harveyi* BB170 bioluminescence assay, indicated that 8 of 30 of the samples contained AI-2. Of the entire wound debridement samples tested, those from DUs most often contained AI-2 (38·5%, Table 3). Debridement samples from VLUs and NHSWs and NHWs least often contained AI-2 (20% and 14·3%, respectively, Table 3). Only two of the 30 debridement samples contained AI-2 and AHLs, and these were from two debridement samples from DUs (Table 2).

Discussion

This work demonstrates that clinical strains of bacteria, that were isolated from different types of chronic wounds, are able to produce cell–cell signalling molecules. Not only can the strains produce cell–cell signalling molecules, when grown under laboratory conditions, but these molecules are likely produced by the bacteria within chronic wounds. Thus, it is possible that these cell–cell signalling molecules mediate interbacterial communication in chronic wound biofilms.

The strains were isolated and randomly chosen for this study from 32 patients suffering from NHSWs, NHWs, DUs, PUs or VLUs. Identification by partial 16S rRNA gene sequencing showed that the strains were from genera that are typically isolated from human chronic wounds (Dowd *et al.* 2008a; James *et al.* 2008). Phylogenetic analysis revealed that clusters of very closely related strains belonging to the same species were isolated and identified from different patients (Table 1 and Fig. 1). Such a finding indicates that specific species are associated with chronic wounds, regardless of their aetiology, and this has been shown by recent ecological studies by other researchers (Hill *et al.* 2003; Davies *et al.* 2004; Dowd *et al.* 2008a,b). *Acinetobacter lwoffii*, *Ser. marcescens* and *Ent. faecalis* strains were isolated from chronic wounds, and strains belonging to these species have been previously isolated from healthy and diseased skin (Roth and James 1988; Berlau *et al.* 1999; Gao *et al.* 2007). *Staphylococcus aureus* are also commonly isolated from both healthy and diseased skin (Gjodsbol *et al.* 2006), although *Staph. aureus* is often found at higher cell densities in wounds (Kirketerp-Moller *et al.* 2008). Strains belonging to *Ac. baumannii* and *Ps. aeruginosa* are commonly isolated from aggressive, rapidly expanding, chronic wounds and evidence suggests that they numerically dominate within most aggressive chronic wounds (Davies *et al.* 2004; Gjodsbol *et al.* 2006). Indeed, *Ac. baumannii* and *Ps. aeruginosa* are considered important wound pathogens and general nosocomial Gram-negative pathogens as they

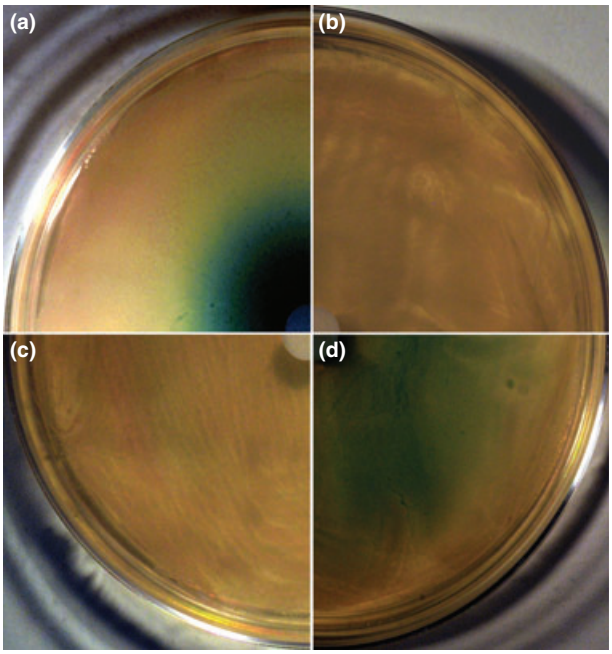

Figure 3 Image of agar plates showing the expression of β-galactosidase activity (blue coloration) by the acyl-homoserine-lactone (AHL) reporter strain *Agrobacterium tumefaciens* A136 to cell-free supernatants from chronic wounds. (a) Strong expression of β-galactosidase during exposure to cell-free batch-culture supernatant from an AHL over-producer *Ag. tumefaciens* A136 (positive control), (b) absence of β-galactosidase activity when exposed to PBS (negative control), (c) absence of activity when exposed to a cell-free chronic wound debridement sample (a negative outcome) and (d) Expression of β-galactosidase during exposure to a cell-free chronic wound debridement sample (positive outcome).

are extremely resistant to antimicrobials (Howell-Jones *et al.* 2005; Navon-Venezia *et al.* 2005; Hernandez 2006; Gootz and Marra 2008) and have a repertoire of mechanisms, such as the expression of surface-attached fimbriae and/or capsular polysaccharide (Pollack 1984; Navon-Venezia *et al.* 2005; Peleg *et al.* 2008), to maintain their presence in chronic wounds. Consequently, these cell populations expand to the detriment of typical resident species within the population (Davies *et al.* 2004).

A comparison of the phylogenetic relatedness of the identified chronic wound strains to their ability to produce cell–cell signalling molecules indicated that members of all of the nine genera, identified in this study, produced either AHL or AI-2 cell–cell signalling molecules. Eight of the 46 strains were not indicated to produce cell–cell signalling molecules. However, for each of these strains, closely related strains belonging to the same genera were shown to produce at last one class or type of signalling molecule (Fig. 1, Table 2). It is possible that observed differences were because of strain-specific timing of cell–cell signalling molecule production or strain-specific differences in the amounts of signalling molecule produced (which is below the level of detection for the observed negative strains). Every genera inferred to produce AHLs and/or AI-2 cell–cell signalling molecules in this research has previously been inferred to be able to produce cell signal molecules by other research groups. However, the majority of the strains tested by other researchers are strains from culture-collections or isolates from environments other than from chronic wounds. Clearly, clinical chronic wound strains share an ability to produce specific cell–cell signal molecules with other strains from the same genera. Also, when considering which strains produced which AHLs or AI-2, *Ser. marcescens*, albeit three of seven of the strains belonging to this genera produced detectable amounts of both classes of cell–cell signalling molecules. The ability to produce both classes of signal molecules has been shown before for this species, and AHLs and AI-2 are known to coordinate biofilm maturation and the production of various extracellular enzymes (Van Houdt *et al.* 2007). The only genera not able to produce AI-2 molecules were those associated with highly aggressive chronic wounds. Specifically, *Ps. aeruginosa* and members of the genera *Acinetobacter* did not produce AI-2 but did produce AHL cell–cell signalling molecules. AHL molecules are known to enhance the biofilm-forming ability of *Ps. aeruginosa* (Juhas *et al.* 2005) and *Acinetobacter* spp. (Niu *et al.* 2008). As such, AHLs may exclusively mediate interbacterial communication system between pathogenic chronic-wound species and facilitate the coordination of activities by groups of pathogenic chronic wound species within healthy (typically resident) multispecies biofilm communities. By coordinating their activities, pathogens will have an improved ability to expand within a biofilm at the expense of the resident community and consequently a chronic wound develops. When considering that *Ag. tumefaciens* A136 was used as a reporter system in this work, it should be remembered that it detects a broad range of AHL signal molecules. Specifically, it is most sensitive to OOHL, *N*-(3-oxo-octanoyl) homoserine lactone and *N*-(3-oxodecanoyl) homoserine lactone. It can also detect longer chains but is insensitive to *N*-butanoyl-L-homoserine lactone, which is one of many AHL molecules of different chain lengths that is also produced by *Ps. aeruginosa* (Williams 2007). *Agrobacterium tumefaciens* A136 is generally considered adequate to study the presence of AHLs in biological samples, because bacteria often produce a broad range of AHLs and many produce long chain AHLs (Erickson *et al.* 2002; Williams 2007). However, the inclusion of a short-chain AHL detector (C4-HSL–C6-HSL) such as *Chromobacterium violaceum* CV026 (McClean *et al.* 1997) could show that samples

that were reported negative for AHLs by *Ag. tumefaciens* A136 actually contain short-chain AHLs.

AHLs and AI-2 were inferred, through bioluminescence and cross-feeding bioassays, to be present in chronic wounds (Table 2). Either of the two classes of molecules were indicated to be present within 21 of 30 (70%) of the chronic wound debridement samples. The method to detect these molecules relied upon the direct sampling of cell-free chronic wound debridement samples, and this contains a complex mixture of human and microbial molecules (Trengove *et al.* 1996; James *et al.* 2000). While not performed in the work reported here, another confirmatory approach to demonstrate that the debridement samples contain bacterial signalling molecules could utilize a solvent extraction method and/or chromatographic techniques to separate and purify bacterial cell–cell signalling molecules from chronic wound debridement samples (Brelles-Marino and Bedmar 2001; Middleton *et al.* 2002; Chambers *et al.* 2005; Nakagami *et al.* 2008). While the use of such protocols has not been successfully used for the purification of AI-2, solvent extraction and chromatographic approaches have demonstrated that AHLs are present in the sputum of cystic fibrosis patients (Middleton *et al.* 2002), in the ruminal contents of cows (Erickson *et al.* 2002), in human food (meat and fish) extracts (Medina-Martinez *et al.* 2007) as well as from pressure-induced ischemic wounds on rats that were infected with *Ps. aeruginosa* (Nakagami *et al.* 2008). In this latter study, Nakagami *et al.* were the first to quantify AHLs from wound samples. Interestingly, they did not detect AHLs in rats with low ($<2{\cdot}0 \times 10^6$ CFU g^{-1}) or high ($>1{\cdot}1 \times 10^8$ CFU g^{-1}) bacterial counts, but only in wounds with bacterial cell concentrations in between these densities. As the authors pointed out, the absence of AHLs at high bacterial densities could be attributed to the degradation (quenching) of AHLs by *N*-acyl-homoserine lactone acylases from *Ps. aeruginosa* (Sio *et al.* 2006), the enhanced production of paraoxonase-like enymes by human cells that hydrolyse the lactone ring of AHLs (Yang *et al.* 2005) as well as the opening of AHL rings in high-pH conditions and changes of temperature (Yates *et al.* 2002). Coincidently, with respect to AI-2, some species of bacteria are also known to internalize AI-2 under specific environmental conditions (Xavier and Bassler 2005; Shao *et al.* 2007; Taga 2007). A similar phenomenon may have occurred during batch culture of *Ent. faecalis* CWS8, where AI-2 concentration apparently dropped in later stationary phase (Fig. 2). Thus, a variety of factors may contribute to changes in cell–cell signal concentration in chronic wounds.

Even though the number of wound debridement samples studied in this work was not extensive (especially when considering the different types of wounds that were sampled), AHLs and AI-2 were only detected twice in the same sample (2 of 30 samples). Such a finding is intriguing and may indicate that either class of molecule could be present at high concentration when specific bacterial species are present in chronic wound biofilms. For example, a shift in chronic wound community composition from an AI-2-producing resident community to that which contains predominantly AHL-producing pathogenic *Ps. aeruginosa* could be accompanied with a change in the dominant type of signal molecule in the wound. With less resident species producing AI-2 and numerically dominant *Ps. aeruginosa* cells producing AHLs, the only detectable class of molecule would be AHLs. This hypothesis must be tempered with the knowledge that, as described earlier, AHLs can be degraded by bacterial and human cells and under certain environmental conditions. Also of consideration are the recent findings by Grice *et al.* (2008) who indicated that between 50% and 60% of healthy skin may contain unculturable *Pseudomonas* spp. This is an interesting finding and, while in contrast to most models that describe the microbial composition of healthy skin, it is possible that indigenous cutaneous *Pseudomonas* spp. (and in particular *Ps. aeruginosa*) respond and coordinate activities via cell–cell signalling following changes in conditions within skin and promote the transition from health to disease. Indeed, while not studied here, an analysis of bacterial composition and species abundance in chronic wounds and comparison to the presence or absence of specific signal molecules could prove to be particularly enlightening and aid in determining a role for signal molecules in wounds. However, at present, available evidence does suggest that cell–cell signalling contributes to the expansion of *Ps. aeruginosa* in burn wounds (Rumbaugh *et al.* 1999; Bielecki *et al.* 2008), although parallel studies of changes in AHL and AI-2 concentrations has yet to be studied in chronic wounds. From a therapeutic standpoint, if cell–cell signalling could be interrupted, for example by quorum sensing interference (Morohoshi *et al.* 2007), then the chronicity of wounds could be reduced.

In conclusion, this study indicates that different clinically relevant chronic wound species produce different classes of cell–cell signal molecules and, for AI-2, is in a growth-phase-dependent manner. Evidence presented here also indicates that these molecules are produced in different types of chronic wounds. Changes in types of cell–cell signal molecule or signal concentration may occur, in part, as a consequence of shifts from an AI-2-producing resident community to that which contain AHL-producing Gram-negative pathogens that hinder wound healing.

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