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Production of Cell-Cell Signaling 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 signaling molecules and (ii) determine if chronic wound debridement samples might contain these molecules.

METHODS AND RESULTS—Partial 16S rRNA gene sequencing revealed the identity of 46 chronic wound strains as 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 produced 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 signaling molecules were detected in 21 of 30 debridement samples of unknown microbial composition.

CONCLUSION—Chronic wound bacteria produce cell-cell signaling molecules. 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 present in human chronic wounds.

SIGNIFICANCE AND IMPACT OF STUDY—Inter-bacterial cell-cell signaling may be an important factor influencing wound development and the presence of AHLs and AI-2 could be used as a predictor of wound severity. Manipulation of cell –cell signaling may provide a novel strategy for improving wound healing.

Keywords

Autoinducer-1; Autoinducer-2; AHL; Biofilm; Signal molecules; Chronic wound

INTRODUCTION

Chronic wounds currently affect 2.5 million to 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 etiology and include

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those related to neuropathic disorders (e.g. diabetic ulcers and diabetic foot ulcers or DUs), vascular disorders (e.g. venous leg ulcers or VLU) and environmentally induced disorders (e.g. pressure ulcers or PUs, non-healing surgical wounds or NHSWs and general chronic non-healing wounds) (Mustoe *et al.*, 2006; Wolcott *et al.*, 2008). While their etiology is distinct, a commonality is the presence of high concentrations of bacteria within the wounds (Wolcott *et al.*, 2008).

Recently, James *et al.* (James *et al.*, 2008) demonstrated that many species of bacteria exist within chronic wounds as part of complex multi-species biofilms. This is a significant finding as bacteria within biofilms exhibit properties that are distinct from planktonic cells (Donlan & 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 & 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 of the normal cutaneous microbial flora, such as *Streptococcus* spp., *Proteus mirabilis*, *Bacillus* spp. and *Staphylococcus* spp. (Dekio *et al.*, 2005; Dowd *et al.*, 2008a; Gjodsbol *et al.*, 2006; James *et al.*, 2008), but also the presence of high cell numbers transient species which are typically Gram-negative rod-shaped bacteria (Lowbury, 1969) such as *Pseudomonas aeruginosa* (Gjodsbol *et al.*, 2006) and *Acinetobacter* spp. (Bowler & Davies, 1999; Hill *et al.*, 2003). The transient species are considered to be highly aggressive chronic wound biofilm species as they are able divide rapidly to numerically dominate within chronic wound multi-species biofilms. Indeed, a recent study by Gjodsbol and coworkers (Gjodsbol *et al.*, 2006) demonstrated that chronic wounds that harbored *P. aeruginosa* within the biofilms were significantly larger than those without *P. aeruginosa*. An ability to control the development of multi-species 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 signaling molecules by species have been repeatedly linked to the enhanced development of single and multi-species biofilms (Irie & Parsek, 2008). A variety of structurally different bacterial cell-cell signaling molecules have been shown to mediate cell-cell communication and include acylated homoserine lactones (AHLs) and autoinducer-2 molecules. AHLs are produced solely by Gram negative bacteria (Williams, 2007) although different species often produced one or more different forms of AHLs. These different AHLs all consist of a homoserine lactone 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 intra-species bacterial communication; different species typically only recognize AHLs produced from closely related species (Miller & Bassler, 2001). However, there are examples where bacterial species can detect AHLs from bacteria belonging to distantly related genera (Bernier *et al.*, 2008; Stickler *et al.*, 1998). Conversely, another class of cell-cell signaling molecule, called autoinducer-2 (AI-2), has been shown to mediate inter-species signaling (Rickard *et al.*, 2006; Surette *et al.*, 1999; Yoshida *et al.*, 2005). AI-2 is an umbrella term used to describe a family of inter-convertible 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- and dual-species biofilm development (Gonzalez Barrios *et al.*, 2006; Hardie & Heurlier, 2008; Rickard *et al.*, 2006).

Recently, work by Nakagami and coworkers (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 *P. aeruginosa* contained AHLs at concentrations up to 0.49 pmol/g. The amount of AHLs increased linearly as the cell density of *P. aeruginosa* in the wound also increased. No AHLs were detected in wounds that were not infected with *P. aeruginosa*. The amount of other cell signaling molecules, such as AI-2, was not determined.

In order to increase understanding of the role of bacterial cell-cell signaling in the development of human chronic wounds, it was the aim of this study to determine if bacteria that are often isolated from typical chronic wound biofilms produce cell-cell signaling molecules. It was also the aim of this work to determine if these signal molecules could be detected in etiologically distinct wounds. We discovered that many different species of chronic wound bacteria produce cell-cell signaling 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, Washington, USA). Strains were randomly chosen from culturable bacterial panels generated from debridement samples taken from patients suffering with diabetic ulcers (DUs), pressure ulcers (PUs), non-healing surgical wounds (NHSWs), non-healing wounds (NHWs) or venous leg ulcers (VLUs). The source of each strain is shown in Table 1. Strains were cultured in Schaedler broth (BD, Franklin Lakes, NJ, USA) supplemented with 1 mM boric acid. Liquid cultures were grown aerobically at 37°C with shaking at 200 rpm.

Vibrio harveyi strains BB170, BB152, BB886 and BB120 (Surette & Bassler, 1998) were used to detect AI-2 (4,5-dihydroxy-2,3-pentanedione) and the AHL N-(3-hydroxybutanoyl) homoserine lactone (HBHL). *Vibrio harveyi* BB170 detects AI-2 while *Vibrio 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 *Vibrio harveyi* BB120 is a producer of HBHL and was used as a positive control. All *V. harveyi* strains were cultured in AB medium (Greenberg *et al.*, 1979) at 30°C. Liquid cultures were grown aerobically at 30°C with shaking at 200 rpm.

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). *A. tumefaciens* KYC6 (Stickler *et al.*, 1998), a producer of OOHL and other AHL molecules, was grown on LB agar at 30°C. *A. 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 μ g/ml) and tetracycline (4.5 g/m) 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, thirty 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 μ L of deionized water was added to the debridement sample. Samples were shaken vigorously using a Qiagen TissueLyser (Qiagen, Valencia, CA) at 30 Hz for 2×30 s. Subsequently, samples were centrifuged at $13,000 \times g$ for 120 s. The supernatant was collected and filtered through a 0.45 μ m Millex® HA filter unit (Millipore, Bedford, MA). Cell-free debridement samples were frozen immediately at -70°C for future use.

Cross-Signaling 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 *A. tumefaciens* A136 was used in a cross-feeding assay developed by Stickler *et al.* (Stickler *et al.*, 1998). LB agar was prepared by covering with 100 μ L of 20 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). *A. tumefaciens* A136 was streaked onto the prepared LB agar and chronic wound strains were streaked in parallel approximately 1 cm away. Plates were then incubated at 30°C for 48 h. If OOH, 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 *A. 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 *A. tumefaciens* A136 colonies blue. Positive and negative controls consisted of culturing the reporter strain with the AHL over-producer strain *A. tumefaciens* KYC6 and with itself (an AHL non-producer).

To determine the presence of a broad range of AHLs in chronic wounds, the AHL detector strain *A. tumefaciens* A136 was also used. To detect AHL cell-cell signaling molecules, 6.5 mm diameter discs of Whatman filter paper (Number One, Whatman, Hillsboro, OR) were impregnated with 5 μ L of cell-free supernatant from chronic wounds. These discs were placed onto the surface of LB agar plates supplemented with 50 μ g/ml spectinomycin and 4.5 μ g/ml tetracycline and pre-coated with 100 μ L of X-Gal (20-mg/ml stock solution in dimethyl formamide) and 100 μ L of a cell-suspension (OD 1.0 at 610 nm) of *A. tumefaciens* A136. Plates were incubated for 72 h at 30°C and 5 μ L cell-free chronic wound supernatant were added to each respective disc every 12 h. The presence of AHLs in wound samples was inferred if the *A. tumefaciens* A136 colonies in contact with the disc or surrounding the disc became blue after 72 h incubation.

Bioluminescence Assays for the Detection of Autoinducer-2 (AI-2) and *V. harveyi* AHL (HBHL) Molecules

HBHL (an AHL that is also referred to as autoinducer-1) and AI-2 activity was determined by testing batch-culture cell-free supernatants using a modification of a bioluminescence assay by Frias *et al.* (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 autoinducer bioassay (AB) medium according to the method of Surette and Bassler (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 analyze 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 mM boric acid. Following 3 h, 6.5 h, 8.5h and 24h batch-culture growth, 1 ml of cultures were collected and passed through a 0.2 μ m filter unit (Fisher Scientific, Suwanee, GA). From these cell-free supernatants, 10 μ L was added to 90 μ L of thawed diluted *V. harveyi* BB170 culture (to detect AI-2) or to 90 μ L of thawed diluted *V. harveyi* BB886 culture (to detect *V. harveyi* HBHL) in a 96-well microplate and analyzed in a Victor 3 multi-label 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 to 1 mM boric acid to growth media not only strengthens the signal from AI-2 producing strains but also enhances background signal (DeKeersmaecker & Vanderleyden, 2003). A comparison to *E. coli* DH5 α , 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 μ l of wound sample was added to 90 μ l of either thawed diluted *V. harveyi* BB170 culture or *V. harveyi* BB886 culture in a 96-well microtitre plate and analyzed in a Victor 3 Victor 3 multi-label counter (Perkin Elmer, Waltham, MA, USA). 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 greater than >10 bioluminescence 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.* (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 μ l of double-autoclaved nanopure water for 7 min and 10 μ l of each boiled suspension were 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 *Escherichia coli* K12 16S rRNA gene sequence. PCR reactions were carried out in PCR Red-Taq reaction buffer (Sigma, St. Louis, MO). 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-quickTM 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 μ l of Big DyeTM (PE Applied Biosystems, Foster City, CA, USA) in a total volume of 20 μ l. 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 (Stackebrandt & 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). Neighbor-joining analysis was conducted with the correction of Jukes and Cantor (Jukes & Cantor, 1969) using TREECON ver. 1.3b (Van de Peer & De Wachter, 1993) with *Thermus thermophilus* (X07998) as the outgroup.

RESULTS

Identification and Phylogenetic Relatedness of Strains

All strains were identified to the genus level and, based upon the criteria of Stackebrandt and Goebel (Stackebrandt & Goebel, 1994), all but two strains (*Klebsiella* sp. CWS47 and *Staphylococcus* sp. CWS31) were speciated (Table 1). The strains belonged to 9 genera and 33 of 46 strains shared $\geq 99\%$ identity to known 16S rRNA gene sequences from previously identified strains that are commonly isolated from chronic wounds. These include *Serratia marcescens*, *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The unspciated strains, *Klebsiella* sp. CWS47 and *Staphylococcus* sp. CWS31, were not given species epithets as they shared an identity of $\leq 97\%$ to the nearest speciated 16S rRNA gene sequence in the EMBL database.

Partial 16S rRNA gene sequence alignment, nearest-neighbor 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 *E. faecalis* CWS36 was identical to *E. faecalis* CWS17, while *E. faecalis* CWS15 was $<98\%$ identical to both *E. faecalis* CWS36 and *E. 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 *Proteus mirabilis*, *S. marcescens*, *P. aeruginosa* and *S. aureus*. Different patients with different wounds could harbor 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 N-(3-hydroxybutanoyl) homoserine lactone (HBHL) (Surette & Bassler, 1998). The second approach used a cross-feeding assay, developed by Stickler *et al.* (Stickler *et al.*, 1998) and relies upon the reporter strain *A. 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 $\leq 0.4\%$ 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 *A. 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 *S. marcescens*, *P. aeruginosa* and *Acinetobacter* spp. were inferred to produce AHL signal molecules (Fig. 1a and b). The reason why AHL cell-cell signal molecules were not detected for all strains belonging to these genera may be due to 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 *A. 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 7 genera were inferred to produce AI-2 (Fig. 1, Table 2). The three highest AI-2 fold-induction values were obtained from batch cultures of *P. mirabilis* CWS11 (382.5 fold induction), *E. faecalis* CWS36 (295.6 fold induction) and *S. 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 *E. faecalis* CWS8 and in batch-cultures of *S. aureus* CWS41 (Fig. 2). *S. 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, *E. 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 extracellularly.

Detection of Cell-Cell Signal Molecules in Chronic Wound Debridement samples

Thirty chronic wound debridement samples were analyzed to determine if AHL and/or AI-2 molecules could be detected. Samples were taken from four etiologically 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 *A. 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). The presence of these AHL molecules was inferred by a change in colour (from off-white to blue) of *A. tumefaciens* A136 colonies in-contact and around cell-free wound debridement sample impregnated discs. In all positive cases, colonies of *A. 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 due to 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 contained AHLs (61.5%, Table 3). Debridement samples from non-healing wounds (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 non-healing wounds (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 signaling molecules. Not only can the strains produce cell-cell signaling 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 signaling molecules mediate inter-bacterial 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 VLU. 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 etiology, and this has been shown by recent ecological studies by other researchers (Davies *et al.*, 2004; Dowd *et al.*, 2008a; Dowd *et al.*, 2008b; Hill *et al.*, 2003). *A. iwoffii*, *S. marcescens* and *E. faecalis* strains were isolated from chronic wounds and strains belonging to these species have been previously isolated from healthy and diseased skin (Berlau *et al.*, 1999; Gao *et al.*, 2007; Roth & James, 1988). *S. aureus* are also commonly isolated from both healthy and diseased skin (Gjodsbol *et al.*, 2006), although *S. aureus* is often found at higher cell densities in wounds (Kirketerp-Moller *et al.*, 2008). Strains belonging to *A. baumannii* and *P. 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, *A. baumannii* and *P. aeruginosa* are considered important wound pathogens and general nosocomial Gram-negative pathogens as they are extremely resistant to antimicrobials (Gootz & Marra, 2008; Hernandez, 2006; Howell-Jones *et al.*, 2005; Navon-Venezia *et al.*, 2005) and have a repertoire of mechanisms, such as the expression of surface-attached fimbriae and/or capsular polysaccharide (Navon-Venezia *et al.*, 2005; Peleg *et al.*, 2008; Pollack, 1984), 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 signaling molecules indicated that members of all of the nine genera, identified in this study, produced either AHL or AI-2 cell-cell signaling molecules. Eight of the 46 strains were not indicated to produce cell-cell signaling molecules. However, for each of these strains, closely related strains belonging to the same genera were shown to produce at least one class or type of signaling molecule (Fig. 1, Table 2). It is possible that observed differences were due to strain-specific timing of cell-cell signaling molecule production or strain-specific differences in the amounts of signaling molecule produced (which is below the level of detection for the observed negative strains). Every genera inferred to produce AHLs and/or autoinducer-2 cell-cell signaling molecules in this research have 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, *S. marcescens*, albeit 3 of 7 of the strains belonging to this genera, produced detectable amounts of both classes of cell-cell signaling 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 extra-cellular 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, *P. aeruginosa* and members of the genera *Acinetobacter* did not produce AI-2 but did produce AHL cell-cell signaling molecules. AHL molecules are known to enhance the biofilm-forming ability of *P. aeruginosa* (Juhas *et al.*, 2005) and *Acinetobacter* spp. (Niu *et al.*, 2008). As such, AHLs may exclusively mediate inter-bacterial communication system between pathogenic chronic-wound species and facilitate the

coordination of activities by groups of pathogenic chronic wound species within healthy (typically resident) multi-species 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 *A. 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 *P. aeruginosa* (Williams, 2007). *A. 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 to C6-HSL) such as *Chromobacterium violaceum* CV026 (McClellan *et al.*, 1997) could show that samples that were reported negative for AHLs by *A. 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 (James *et al.*, 2000; Trengove *et al.*, 1996). While not performed in the work reported here, another confirmatory approach to demonstrate that the debridement samples contain bacterial signaling molecules could utilize a solvent extraction method and/or chromatographic techniques to separate and purify bacterial cell-cell signaling molecules from chronic wound debridement samples (Brelles-Marino & Bedmar, 2001; Chambers *et al.*, 2005; Middleton *et al.*, 2002; Nakagami *et al.*, 2008). While the use of such protocols have 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 *P. aeruginosa* (Nakagami *et al.*, 2008). In this latter study, Nakagami and coworkers were the first to quantify AHLs from wound samples. Interestingly, they did not detect AHLs in rats with low ($< 2.0 \times 10^6$ CFU/g) or high ($> 1.1 \times 10^8$ CFU/g) bacterial counts. 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 *P. aeruginosa* (Sio *et al.*, 2006), the enhanced production paraoxonase-like enzymes by human cells that hydrolyze the lactone ring of AHLs (Yang *et al.*, 2005) and opening of AHL ring in high pH conditions and changes of temperature (Yates *et al.*, 2002). Coincidentally, some species of bacteria are also known to internalize AI-2 under specific environmental conditions (Shao *et al.*, 2007; Taga, 2007; Xavier & Bassler, 2005). A similar phenomenon may have occurred during batch culture of *E. 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 is fairly small (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 *P. 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 *P. 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, AHL and AI-2 can be degraded by bacterial and human cells and under certain environmental conditions. 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 signaling contributes to the expansion of *P. aeruginosa* in burn wounds (Bielecki *et al.*, 2008; Rumbaugh *et al.*, 1999), 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 signaling could be interrupted, for example by QS 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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Fig. 1.

Neighbor-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 10 nucleotides. Sequenced chronic wound strains are labeled with the identifier CWS followed by a strain number and most-likely identities are assigned. Colors represent the inferred production of AHL or AI-2 cell-cell signal molecules by that strain. Strains labeled with red text are those that produce AI-2 (using the reporter strain *V. harveyi* BB170) while

green boxes label those strains that produce AHL molecules (using the reporter strain *A. tumefaciens* A136). Text of strains that are only colored grey represents strains that did not produce detectable quantities of either signal molecule. None of the strains produced HBHL 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.

Fig. 2. Kinetics of growth and 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 *V. 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.

Fig. 3. Image of agar plates showing the expression of β -galactosidase activity (blue coloration) by the AHL reporter strain *A. tumefaciens* A136 to cell-free supernatants from chronic wounds. (A) Production β -galactosidase during exposure to cell-free batch-culture supernatant from an AHL overproducer *A. 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) Production of β -galactosidase (positive outcome) during exposure to a cell-free chronic wound debridement sample.

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	<i>Staphylococcus aureus</i>	753	98	<i>Staphylococcus aureus</i>	FM207477
NHW	CWS34	<i>Staphylococcus aureus</i>	750	99	<i>Staphylococcus aureus</i>	FM207534
PU	CWS30	<i>Staphylococcus aureus</i>	748	98	<i>Staphylococcus aureus</i>	FM207530
PU	CWS31	<i>Staphylococcus aureus</i>	758	97	<i>Staphylococcus</i> sp.	FM207531
PU	CWS41	<i>Staphylococcus aureus</i>	765	99	<i>Staphylococcus aureus</i>	FM207541
VLU	CWS37	<i>Staphylococcus aureus</i>	751	100	<i>Staphylococcus aureus</i>	FM207537
DU	CWS5	<i>Serratia marcescens</i>	755	98	<i>Serratia marcescens</i>	FM207481
DU	CWS35	<i>Serratia marcescens</i>	759	98	<i>Serratia marcescens</i>	FM207535
NHW	CWS39	<i>Serratia marcescens</i>	751	99	<i>Serratia marcescens</i>	FM207539
NHW	CWS44	<i>Serratia marcescens</i>	753	99	<i>Serratia marcescens</i>	FM207544
PU	CWS40	<i>Serratia marcescens</i>	741	99	<i>Serratia marcescens</i>	FM207540
VLU	CWS4	<i>Serratia marcescens</i>	752	99	<i>Serratia marcescens</i>	FM207480
VLU	CWS25	<i>Serratia marcescens</i>	751	100	<i>Serratia marcescens</i>	FM207962
DU	CWS7	<i>Pseudomonas aeruginosa</i>	750	99	<i>Pseudomonas aeruginosa</i>	FM207483
DU	CWS12	<i>Pseudomonas aeruginosa</i>	698	99	<i>Pseudomonas aeruginosa</i>	FM207514
DU	CWS13	<i>Pseudomonas aeruginosa</i>	747	99	<i>Pseudomonas aeruginosa</i>	FM207515
DU	CWS14	<i>Pseudomonas aeruginosa</i>	712	99	<i>Pseudomonas aeruginosa</i>	FM207516
PU	CWS29	<i>Pseudomonas aeruginosa</i>	742	98	<i>Pseudomonas aeruginosa</i>	FM207529
PU	CWS33	<i>Pseudomonas aeruginosa</i>	710	98	<i>Pseudomonas aeruginosa</i>	FM207533
VLU	CWS3	<i>Pseudomonas aeruginosa</i>	748	99	<i>Pseudomonas aeruginosa</i>	FM207479
NHW	CWS22	<i>Proteus mirabilis</i>	748	99	<i>Proteus mirabilis</i>	FM207524
NHW	CWS27	<i>Proteus mirabilis</i>	748	99	<i>Proteus mirabilis</i>	FM207527
PU	CWS23	<i>Proteus mirabilis</i>	738	99	<i>Proteus mirabilis</i>	FM207525
PU	CWS48	<i>Proteus mirabilis</i>	791	100	<i>Proteus mirabilis</i>	FM207476
VLU	CWS11	<i>Proteus mirabilis</i>	750	99	<i>Proteus mirabilis</i>	FM207513
VLU	CWS26	<i>Proteus mirabilis</i>	744	99	<i>Proteus mirabilis</i>	FM207526
NHSW	CWS38	<i>Klebsiella pneumoniae</i>	742	100	<i>Klebsiella pneumoniae</i>	FM207538
NHW	CWS45	<i>Klebsiella pneumoniae</i>	779	99	<i>Klebsiella pneumoniae</i>	FM207545
NHW	CWS47	<i>Klebsiella pneumoniae</i>	713	96	<i>Klebsiella</i> sp.	FM207963

Wound type	Strain	Most closely related species or genus	Sequence length (bp)	Identity (%)	Assigned identity	EMBL accession number
VLU	CWS42	<i>Klebsiella oxytoca</i>	748	98	<i>Klebsiella oxytoca</i>	FM207542
VLU	CWS19	<i>Escherichia coli</i>	743	98	<i>Escherichia coli</i>	FM207521
VLU	CWS32	<i>Escherichia coli</i>	749	100	<i>Escherichia coli</i>	FM207532
DU	CWS6	<i>Enterococcus faecalis</i>	766	99	<i>Enterococcus faecalis</i>	FM207482
DU	CWS15	<i>Enterococcus faecalis</i>	702	98	<i>Enterococcus faecalis</i>	FM207517
DU	CWS17	<i>Enterococcus faecalis</i>	743	100	<i>Enterococcus faecalis</i>	FM207519
NHSW	CWS36	<i>Enterococcus faecalis</i>	772	100	<i>Enterococcus faecalis</i>	FM207519
NHW	CWS46	<i>Enterococcus faecalis</i>	769	99	<i>Enterococcus faecalis</i>	FM207546
PU	CWS8	<i>Enterococcus faecalis</i>	736	99	<i>Enterococcus faecalis</i>	FM207484
PU	CWS16	<i>Enterococcus faecalis</i>	730	98	<i>Enterococcus faecalis</i>	FM207518
VLU	CWS10	<i>Enterococcus faecalis</i>	743	99	<i>Enterococcus faecalis</i>	FM207512
VLU	CWS21	<i>Enterococcus faecalis</i>	754	99	<i>Enterococcus faecalis</i>	FM207523
NHSW	CWS43	<i>Bacillus mojavensis</i>	757	99	<i>Bacillus mojavensis</i>	FM207543
NHW	CWS28	<i>Acinetobacter iwoffii</i>	733	99	<i>Acinetobacter iwoffii</i>	FM207528
VLU	CWS9	<i>Acinetobacter calcoaceticus</i>	730	98	<i>Acinetobacter calcoaceticus</i>	FM207485
VLU	CWS20	<i>Acinetobacter calcoaceticus</i>	737	99	<i>Acinetobacter calcoaceticus</i>	FM207522
VLU	CWS18	<i>Acinetobacter baumannii</i>	735	99	<i>Acinetobacter baumannii</i>	FM207520

Table 2

Induction of cell-cell signal reporter strains by chronic wound strains. Positive results are highlighted in bold. Where values are decimals, the nearest integer is considered.

Strain	<i>A. tumefaciens</i> A136	<i>V. harveyi</i> BB886	<i>V. harveyi</i> BB170
<i>Vibrio harveyi</i> BB152*	nd	nd	631.6
<i>Vibrio harveyi</i> BB120**	nd	2776.2	nd
<i>Escherichia coli</i> DH5a***	–	0.4	12.8
<i>Staphylococcus aureus</i> CWS1	–	1.4	33.2
<i>Staphylococcus aureus</i> CWS34	–	0.3	137
<i>Staphylococcus aureus</i> CWS30	–	0.3	164.9
<i>Staphylococcus sp.</i> CWS31	–	0.3	23.3
<i>Staphylococcus aureus</i> CWS41	–	0.3	75.6
<i>Staphylococcus aureus</i> CWS37	–	0.2	24.7
<i>Serratia marcescens</i> CWS5	+	1.8	271.6
<i>Serratia marcescens</i> CWS35	–	1.3	30.1
<i>Serratia marcescens</i> CWS39	–	0.1	30.4
<i>Serratia marcescens</i> CWS44	+	9.6	134.5
<i>Serratia marcescens</i> CWS40	–	3	35.4
<i>Serratia marcescens</i> CWS4	+	2.2	205.3
<i>Serratia marcescens</i> CWS25	–	2.3	45.5
<i>Pseudomonas aeruginosa</i> CWS7	–	1	4.3
<i>Pseudomonas aeruginosa</i> CWS12	+	0.5	13.7
<i>Pseudomonas aeruginosa</i> CWS13	+	2.8	2.16
<i>Pseudomonas aeruginosa</i> CWS14	+	0.3	11
<i>Pseudomonas aeruginosa</i> CWS29	–	0.5	0.57
<i>Pseudomonas aeruginosa</i> CWS33	+	0	1.02
<i>Pseudomonas aeruginosa</i> CWS3	–	0.2	10.3
<i>Proteus mirabilis</i> CWS22	–	0.8	110
<i>Proteus mirabilis</i> CWS27	–	2.6	69.4
<i>Proteus mirabilis</i> CWS23	–	1.5	133
<i>Proteus mirabilis</i> CWS48	–	0.2	44.4
<i>Proteus mirabilis</i> CWS11	–	2.6	382.5
<i>Proteus mirabilis</i> CWS26	–	1.9	63.3
<i>Klebsiella pneumoniae</i> CWS38	–	0.1	30.7
<i>Klebsiella pneumoniae</i> CWS45	–	1.1	168.3
<i>Klebsiella sp.</i> CWS47	–	0.7	174
<i>Klebsiella oxytoca</i> CWS42	–	0.2	44.4
<i>Escherichia coli</i> CWS19	–	1.3	196.1
<i>Escherichia coli</i> CWS32	–	8.8	99.3
<i>Enterococcus faecalis</i> CWS6	–	0.5	165.7
<i>Enterococcus faecalis</i> CWS15	–	1.6	29.8
<i>Enterococcus faecalis</i> CWS17	–	2.0	182.3

Strain	<i>A. tumefaciens</i> A136	<i>V. harveyi</i> BB886	<i>V. harveyi</i> BB170
<i>Enterococcus faecalis</i> CWS36	–	5.2	295.6
<i>Enterococcus faecalis</i> CWS46	–	0.6	201.5
<i>Enterococcus faecalis</i> CWS8	–	1.1	191.5
<i>Enterococcus faecalis</i> CWS16	–	0.2	30.8
<i>Enterococcus faecalis</i> CWS10	–	0.7	89.3
<i>Enterococcus faecalis</i> CWS21	–	2	19.82
<i>Bacillus mojavensis</i> CWS43	–	1.2	40.74
<i>Acinetobacter lwoffii</i> CWS28	–	0.8	0.75
<i>Acinetobacter calcoaceticus</i> CWS9	–	1.9	3.4
<i>Acinetobacter calcoaceticus</i> CWS20	+	0.7	1.7
<i>Acinetobacter baumannii</i> CWS18	+	1.2	4.2

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

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

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), non-healing surgical wounds (NHSWs), non-healing wounds (NHWs) or venous leg ulcers (VLUs). Bold symbols or values are positive results.

<i>Wound sample</i>	<i>Sample weight (mg)</i>	<i>A. tumefaciens</i> A136	<i>V. harveyi</i> BB886	<i>V. harveyi</i> 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