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CELL-TO-CELL COMMUNICATION IN *SALMONELLA TYPHIMURIUM* DT104

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Abstract

Salmonella is a genus of the family *Enterobacteriaceae*. Members belonging to this family are characterised as Gram-negative, non-spore forminer, rod-shaped bacteria. Motility forms of these bacteria have peritrichous flagella. Many members of this family of bacteria are commonly located in the intestinal tract of both animals and humans as either pathogens or commensals. The aim of this study was to confirm the absence or presence of homoserine lactones for the purpose of quorum sensing. Homoserine lactones are single molecules that are expressed by the bacteria when a critical cell mass has been reached. It allows the bacteria to coordinate their activities and survive adverse conditions. Bacterium isolated from clinical source was screened using thin layer chromatography technique. The indicator strains in used were *Chromobacterium violaceum* CV026 and *Agrobacterium tumifaciens* NTI, the control strain was *Pseudomonas aerugonisa* PAO1. Results demonstrated that using Thin Layer Chromatography (TLC), the clinical isolate, *Salmonella tympuimurium* DT104 tested positive for both short chain and long chain acyl homoserine lactones.

Key words: *Salmonella tympuimurium* DT104, Biofilm, quorum sensing

Introduction

Salmonella continue to be prominent in the global food chain (Fisher *et al*. 2002; Werber *et al*. 2002), as it is frequently isolated from raw meats, milk and milk products, poultry processing equipment particularly in the slaughter and evisceration area, each of which have been associated with outbreaks of *Salmonella* (Bonner *et al*., 2001; Joseph *et al*., 2001; Ryan *et al*., 1997; Tauxe, 1997; Hennessy *et al*., 1996). There is little evidence to date to suggest the presence of Salmonella in biofilm formation; however, the poultry processing operation is an ideal environment for biofilm formation as a wet atomosphere. A variety of studies have confirmed that *Salmonella* can actually attach to surfaces found in food processing plants, including plastic, cement and stainless steel and form a biofilm (Jones and Bradshaw, 1997).

Quorum sensing is a cell-to-cell communication system used by bacteria (Parsek, *et al* 1999). It is a system in which an autoinducer accumulates in the extracellular

environment, which allows bacteria to sense their population density (Fuqua *et al*., 1996). Once a certain population density is reached a process termed Autoinduction occurs, which coordinately expresses selected genes in response to the extra cellular concentration of the autoinducer (Flavier *et al*, 1997).

Different bacterial populations have been shown to use quorum sensing mechanisms to regulate a broad range of biological functions (Lin *et al*., 2003), including bioluminescence, virulence factor production, antibiotic and secondary metabolite production, the ability to move by swarming and biofilm formation (Fuqua *et al*., 1996) as well as the induction of stationary phase responses in individual bacteria (Withers *et al*., 2001).

The ability of bacteria to respond to autoinducers in their environment gives them the advantage of only performing these important biological functions when living in a community. Quorum sensing is therefore used by bacteria to direct the activities of the community, allowing bacteria within a population to take on some of the features of multicellular organisms (Taga and Bassler, 2003). It is used by numerous microorganisms living in distinct environments ranging from the gastrointestinal tract of animals to sewage fluid may confer a strong selective advantage upon relevant microorganisms living in diverse habitats (Schaefer *et al*., 1996).

There are two types of bacterial quorum sensing systems, Gram-negative bacteria LuxIR circuits and Gram-positive oligopeptide two component circuits (Taga and Bassler, 2003).

Thin layer chromatography is a simple, rapid and sensitive assay for analysing bacterial cultures for the presence of *N*-acyl-homoserine lactones (AHLs) (Shaw *et al*., 1997). The assay can be used to screen many samples simultaneously (Shaw *et al*., 1997). A reporter strain is required to visualise the assay following the development of the plate. These monitoring systems involve a phenotypic response e.g. bioluminescence, violacin production or β-galactosidase activity, activated through an AHL –receptor protein (Ravn *et al*., 2001). Biological assays facilitates the screening of microorganisms for AHL molecules as well as increasing the rate of identification of lux I proteins responsible for their synthesis (Pearson *et al*., 1994). One indicator bacterium is *Chromobacterium violaceum* CV026. *Chromobacterium violaceum* can be used in both the well diffusion assay and thin layer chromatography as overlays to detect a diverse selection of AHL molecules (McClean *et al*., 1997). The CviR of *Chromobacterium violaceum* regulates the

production of violacein, a purple pigment when induced by AHL's (Ravn *et al*., 2001). The purple pigment is water insoluble and has antibacterial activity (McClean *et al*., 1997). The CviR of *Chromobacterium violaceum* responds mainly to short chain unsubstituted AHL's with Acyl side chains of 4 to 8 carbons in length (McClean *et al*., 1997).

Therefore it was decided to investigate whether *S. typhimurium* DT104 produces HSLs. As *S. typhimurium* DT104 is regarded as a pathogen of low virulence and quorum sensing is often linked to virulence gene expression.

Materials and Methods

Detecting *N*-Acyl-homoserine lactone quorum sensing signals produced by *Salmonella tympuimurium* DT104

Acyl-homoserine lactone Monitor Strains

The indicator strain *Chromobacterium violaceum* CV026 with 20µg/ml Kanamycin or *Agrobacterium tumifaciens* NTI with 20µg/ml Gentamicin, were grown in Luria Bertoni (LB) broth and incubated over night at 30^{o}C with aeration. One milliter of this culture was added to 300 ml LB broth containing 1% agar (LBA) at 42^{o}C. This media/ culture mix was overlaid on top of the thin layer chromatography (TLC) plate in a sterile pyrex dish until the entire plate is covered.

Extraction of culture supernatant for thin layer chromatography

Forty-milliliter culture volumes were centrifuged at 10,000g for 10 minutes at 4^{o}C and the cells were removed by centrifugation, supernatants were extracted twice with equal volumes of dichloromethane, the mixture was shaken vigorously for 30 second and the phases were allowed to separate. The shaking was repeated three times before the dichloromethane containing fraction was removed and other 40-ml fraction added and the combined extracts were dried over anhydrous magnesium sulphate, filtered using a filter paper Whatman No. 1 qualitative filter. The whole extraction process was repeated three times. The combined dichloromethane were evaporated at 40ºC to dryness and reconstituted in 2 ml dichloromethane, transfer to an HPLC-vial, dried under nitrogen flow and stored at –20^{o}C after a final reconstitution in between 0.3ml acetonitrile (Ravn *et al*., 2001; Shaw *et al*., 1997).

Thin Layer Chromatography (TLC)

Between 1-25µl of HSL extractions, dilutions of extractions AHL standard were applied to a TLC plate and the plate developed in 60:40 (v/v) methanol/ water or

acetone/hexane 55:45 (vol/vol) until the solvent front reached the top of the plate (approx. takes 4 hours for RP-18F_{254S} or 70 minutes for a Silica gel 60F_{254}), the plate was removed from the chromatography tank. The TLC plate was allowed to dry for at least 10 minutes while an agar cover layer was prepared of either *Agrobacterium tumifaciens* NTI with 60µg/ml of 5-Bromo-4-Chloro-3-Indolyl β-D-galactopyranoside (X-Gal) or *Chromobacterium violaceum* CV026 (indicator bacteria). The agar top-layers were prapered as for the well diffusion assay and the culture-agar solution immediately poured on the top of the TLC-plate. After the agar solidified, the coated plates were incubated at 28^{o}C for 24 hours in a closed glass container (Ravn *et al*., 2001; Shaw *et al*., 1997).

Results

Detection of short chain homoserine lactone

From the results obtained (Figure 1), it may be concluded that *Salmonella typhimurium* DT104 is actually capable of producing homeserine lactones for the purpose of quorum sensing. In Figure 1, the test, *Salmonella typhimurium* DT104 clinical isolate, was demonstrated to successfully produce acyl-homoserine lactones (HSLs) on carbon 4 and carbon 6 of the acyl side chain. The arrows indicate the spots on the agar to which the acyl-HSL has migrated to the following TLC step.

The expression of the purple pigment released by the indicator strain confirms the presence of these signal molecules in the test bacteria. *Pseudomonas aerugonisa* PAO1, a violet spot was also observed in its lane, which confirmed that PAO1 induced violaceum production in the positive control.

Detection of long chain homoserine lactone

The results showed that the *Salmonella typhimurium* DT104 clinical isolate, gave positive production of a C12-HSL signal molecule compared to the positive control strain *P. aeruginosa* PAO1 and C12-HSL (Sigma) standard (Figure 2).

The *Agrobacterium tumifaciens* NTI strain does not produce its own signal molecule. Therefore it is a suitable bacterium to utilize for the detection of long chain acyl-HSLs. The presence of an acyl-HSL was confirmed on carbon 12 of the acyl side chain following TLC. The results demonstrate that locations to which an active compound has migrated on the plate yielded a blue zone, resulting from the hydrolysis of the X-gal in the medium.

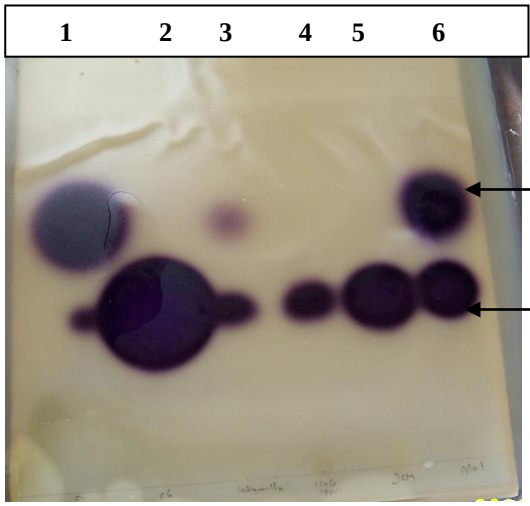

Figure 1: TLC plates RP-18F$_{254S}$ overlaid with *C. violaceumm* CV026. The chromatograms were developed with methanol/water (60:40 v/v) following development, the solvent was evaporated by allowing the TLC plate to dry for 10 minutes.
Lane 1: C4 (2mM), Lane 2: C6 (25mM) HSL (Sigma) 2 µl; Lane 3: *Salmonella typhimurium* DT104 (25 µl); Lane 4 and 5: gram negative bacteria; Lane 6: *P. aeruginosa* PAO1 positive control 20 µl

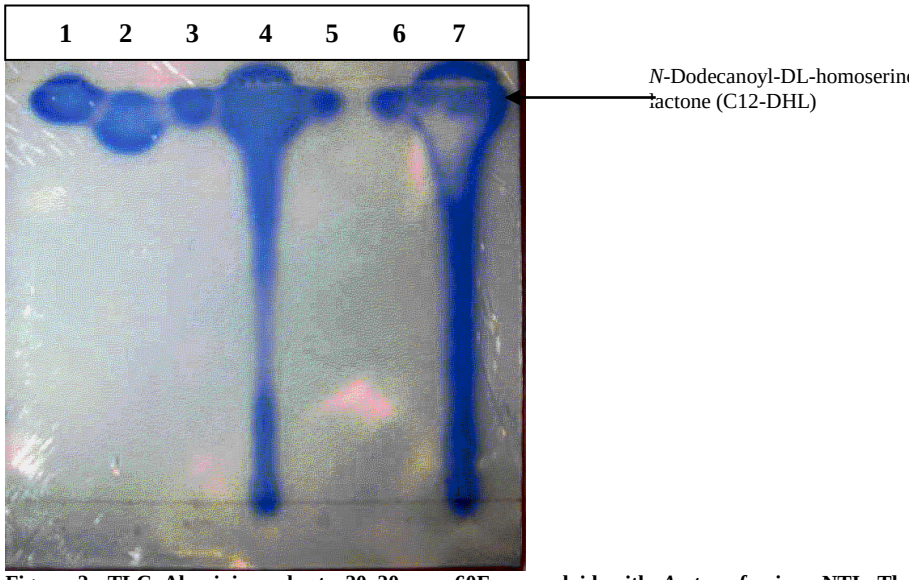

Figure 2: TLC Aluminium sheets 20x20 cm, 60F$_{254}$ overlaid with *A. tumefasciens* NTI. The chromatograms were developed with acetone/hexane (55:45 v/v) following development the solvent was evaporated by allowing the TLC plate to dry for 10 minutes.
Lane 1: C12-HSL (Sigma) 5 µl (2mM); Lane 2 and 3: gram negative bacteria; Lane 4: *Salmonella tympuimurium* DT104; Lane 5 and 6: gram negative bacteria; Lane 7: *P. aeruginosa* PAO1 positive control 7 µl

Discussion

Using *Chromobacterium violaceum* CV026 and *Agrobacterium tumifaciens* NTI as the indicator bacteria strain it was found that *Salmonella typhimurium* strain induce violacin production and consequently produce C_4, C_6 and C_{12} HSLs. In the case of *Agrobacterium tumifaciens* NTI of this study, this bacterial indicator responds to 3-oxo-substituted HSL derivates with chain lengths 12 carbons. The *Chromobacterium violaceum* CV026 reporter assay was used to detect short chain acyl-HSL varying from 4 to 6 carbons. The stimulation or inhibition for violaceum production in *Chromobacterium* can be usefully employed in thin layer chromatography overlays to detect a structurally diverse range of both natural and synthetic acyl HSLs. Both of the assays described in this experiment were supplemented with antibiotic, the bio-indicator *Agrobacterium tumifaciens* NTI was supplemented with 50µl of the antibiotic Gentamycin, and the *Chromobacterium violaceum* CV026 was supplemented with 50µl of the antibiotic Kanamycin. The purpose of this step was to ensure the prevention of contamination and gurantee plasmid stability. In addition to this, the *Agrobacterium tumifaciens* NTI was further supplemented with 150µl of 5-bromo-4-chloro-3-indoly β-D-galactopyranosyl (X-gal).

To date, very little research has been dedicated to investigating cell-to-cell communication in *Salmonella typhimurium* DT104. Surette and Bassler (1998) have investigated cell-to-cell communication using the marine bacterium *Vibrio harveyi* system. Dalton *et al*., (1994) have studied the factors that affect the microbial attachment to abiotic surface, and it was discovered that cell envelope, whose chemistry may change in respone to environmental stimuli and quorum sensing, influences adherent properties of the cell. Test bacteria, such as *Staphylococcus aureus, Eschericha coli* and *Salmonella epidermis,* demonstrated that the genes responsible for surface protein expression, attachment and extracellular polymeric substances are activated in response to stimuli, such as population density, stress or nutrient limitations. There is little information on the presence of *Salmonella* in biofilms. However, Joseph *et al*., 2001, demonstrated that *Salmonella* is capable of attaching and forming a biofilm on surfaces such as plastic, cement and stainless steel. There are obvious environmental implications of this bacterium to survive adverse conditions and form these structured communities, especially on stainless steel. This may pose numerous hazards, especially in the food processing industries, as it allows *Salmonella* to proliferate and spread to virtually to all parts of the factory.

Conclusion

The homoserine lactones (HSLs) detection experiment showed that *Salmonella typhimurium* strain produce short chain and long chain HSLs detectable by the indicator bacteria *Chromobacterium violaceum* CV026 and *Agrobacterium tumifaciens* NTI. On completion of this set of experiments, it has been clearly demonstrated that *Salmonella typhimurium* DT104, clinical isolate, is capable of producing homoserine lactones. There are obvious advantages for cell-to-cell communication in this species, as it allow proliferation and survival under adverse conditions. The possibility of this strain of bacteria forming a biofilm poses many adverse effects both on food and industries.

Further research into this topic is essential. Results from this study confirm that *Salmonella typhimurium* DT104 has the ability to communicate intracellularly, which may be a causative factor for the development of increased resistance to antibiotic intervention. This has vast implications, which need further exploration in order to understand the mechanisms and processes involved in signalling cascades.

Another research issue that may be important to address is the ability of *Salmonella* to form biofilms on various materials, especially those that are used on a daily basis in a number of industries, which may pose potential health hazards to humans. Due to time constraints, this area of research firmly believes that it is necessary.

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السالمونيلا هي بكتيريا من عائلة *Enterobacteriaceae* و توصف هذه العائلة هو أنها بكتيريا سالبة لصبغة الجرام ولا تكون أبواغا و قصيبية الشكل ،متحركة بواسطة أسواط موزعة على جسم الخلية . العديد من أفراد عائلتها تتواجد عادة في أمعاء الحيوانات و الإنسان كجراثيم مسببة للأمراض . الهدف من هذه الدراسة هو إثبات وجود أو عدم وجود homeserine lactones بهدف استشعار النصاب . homoserine lactones هي جزيئات أحادية يعبر عنها بالبكتيريا عند الحصول على كتلة خلايا حرجة وهي تمكن البكتيريا من تنسيق نشاطاتها والبقاء على قيد الحياة في الظروف الصعبة . تم عزل بكتيريا *Salmonella typhimurium* DT104 من مصدر مختبري ومن ثم تم فحص وجود أو عدم وجود homoserine lactones من خلاياها باستخدام طريقة الطبقة الرقيقة الكروموتوغرافية (TLC) . كانت السلالات الاستدلالية المستخدمة هي (*Chromobacterium violaceum* CV026) و (*Agrobacterium tumifaciens* NTI) و كانت بكتيريا التحكم الإيجابية هي(*Pseudomonas aerugonisa* PAO1) . أثبتت النتائج أنه باستخدام طريقة الطبقة الرقيقة الكروموتوغرافية (TLC) أن البكتيريا *Salmonella typhimurium* DT104المعزولة من مصدر مختبري تحتوي على سلاسل من homoserine lactones .