Altering Substrate Chain Length Specificity of an Acylhomoserine Lactone Synthase in Bacterial Communication*[^S]

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Quorum sensing mediated by specific signal compounds (autoinducers) allows bacteria to monitor their cell density and enables a synchronized regulation of target gene sets. The best studied group of autoinducers are the acylhomoserine lactones (AHSLs), which are central to the regulation of virulence in many plant and animal pathogens. Variation of the acyl side chain of the AHSLs underlies the observed species specificity of this communication system. Here we show that even different strains of the plant pathogen Erwinia carotovora employ different dialects of this language and demonstrate the molecular basis for the acyl chain length specificity of distinct AHSL synthases. Under physiological concentrations, only the cognate AHSL with the “right” acyl chain is recognized as a signal that will switch on virulence genes. Mutagenesis of the AHSL synthase gene expI[SCC1] identified the changes M127T and F69L as sufficient to effectively alter ExpI[SCC1] (an N-3-oxohexanoyl-l-homoserine lactone producer) substrate specificity to that of an N-3-oxooctanoyl-l-homoserine lactone producer. Our data identify critical residues that define the size of the substrate-binding pocket of the AHSL synthase and will help in understanding and manipulating this bacterial language.

Bacteria are able to monitor their cell density by producing and detecting specific signal compounds commonly referred to as autoinducers. This quorum sensing allows bacteria to respond to fluctuations in their numbers and enables synchronous regulation of target gene sets when living in a community. The best studied group of autoinducers are the acylhomoserine lactones (AHSLs),[^1] which are central to the regulation of virulence in Gram-negative plant and animal (including human) pathogens (1–5) and have been shown to control processes as diverse as bioluminescence in the marine organism Vibrio Fischeri (6), biofilm formation and virulence in the opportunistic human pathogen Pseudomonas aeruginosa (7, 8), Ti plasmid conjugal transfer in Agrobacterium tumefaciens (9, 10), production of the exopolsaccharide stewartian acting as a virulence factor in the maize pathogen Pantoaea stewartii (11), and production of carbapenem antibiotics as well as plant cell wall-degrading extracellular enzymes in the plant pathogen Erwinia carotovora (12, 13). The chain length (C4–C18) and the oxidative status of the acyl side chain of the AHSLs vary and reflect the observed species-specificity of this communication system (2, 4).

However different these processes are, the general mechanism of autoinducer-mediated quorum sensing is similar and involves an AHSL synthase and a sensor protein binding to the cognate autoinducer and regulating target gene expression. The I-protein produces basal levels of a specific AHSL, which passes through bacterial cell membranes by diffusion and/or active transport (2, 5). After the AHSL concentration reaches a critical threshold level corresponding to a certain quorum of bacteria, the AHSL interacts with the cognate R-protein and controls the expression of quorum sensing-regulated target genes (1, 2, 14). At present, >70 AHSL synthases (LuxI-like I-proteins) and sensors (LuxR-like R-proteins) are known (4). In E. carotovora, the production of both carbapenem antibiotics and extracellular enzyme (cellulase, pectate lyase, polygalacturonase, and protease) virulence factors is controlled by AHSLs and, hence, requires a functional I-protein known as CarI or ExpI, respectively (1, 13, 14). Although carbapenem synthesis in E. carotovora ATT39048 is positively regulated by an R-protein (CarR) (1, 15), the cognate AHSL sensor protein and the mechanism for extracellular enzyme regulation is not known (1, 14, 16). For the actual change in target protein levels, several other factors might be important, including small regulatory RNA chaperones influencing RNA stability (e.g. Hfq of Vibrio harveyi and RsmA in E. carotovora (17, 18) and proteins binding to R-proteins (e.g. TraM of A. tumefaciens) (19).

The enzymatic reaction mechanism of AHSL synthesis involves the substrates S-adenosyl-l-methionine and an acyl-acetyl carrier protein (acyl-ACP) (20–22). The crystal structure of EsI, the I-protein of P. stewartii, revealed a high similarity of the AHSL synthase to N-acetyltansferases and allowed modeling of the 3-oxo-C6-phosphopantetheine part of acyl-ACP in the active cavity of EsI. Mutation analysis was used to demonstrate the importance of several residues for the activity of EsI, thereby confirming the model (23). Additional conserved residues essential for activity have been identified in LuxI (24) and RhlII (25). In EsI, the substrate acyl-ACP selection seems to be due to binding to a hydrophobic pocket. Indeed, changing of the conserved Thr-140 to Ala resulted in the production of
fully reduced homoserine lactones instead of N-3-oxohexanoyl-L-homoserine lactone (3-oxo-C6-HSL), which has been explained as a stabilizing effect of a favorable hydrogen bond between the C3 carbonyl and the hydroxy group of Thr (23). The recently described crystal structure of LasI from P. aeruginosa seems to indicate that different bacteria might have different mechanisms for substrate selection. Here, a tunnel with no apparent restriction on acyl chain length passing through the enzyme can accommodate the acyl chain of the acyl-ACP substrate (26).

It has been speculated (2, 23) that the C terminus of I-proteins should contain residues responsible for specificity to a substrate with a certain acyl side chain length, but the actual residues and structural prerequisites determining this specificity have not yet been identified. Here, we describe the specificity of AHSL signaling in E. carotovora and demonstrate the molecular basis for the substrate chain length specificity of the AHSL synthase ExpI. The changes M127T and F69L in ExpI resulted in a product shift from 3-oxo-C6-HSL to N-3-oxoctanoyl-L-homoserine lactone (3-oxo-C8-HSL). Additional multiple amino acid changes restored production of the changed product 3-oxo-C8-HSL to wild type levels.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Mutagenesis**—The E. carotovora strains SCC1 and SCC3193 originate from potato field collections near the Viikki Campus of the University of Helsinki (27). The expI** gene was amplified from E. carotovora SCC1 DNA by PCR with 5′-GGGCTAC- CATGTTAGATTTG-3′ (5′-GGGCTACCATGTTAGATTTGATTTG- TAAATC′- without ATG for cloning into pQE30 (Qiagen)) and 5′-GGAACTTTCAAGCTTGAAGATTTG-3′ cloned into BamHI/HindIII sites of pBluescript II SK or pQE30 and transformed into Escherichia coli DH5α and JM109. ExpI** (GenBank™ accession number X59565) was >98% identical to Car and 70% identical to ExpI. Mutations were created using the Stratagene QuikChange site-directed mutagenesis kit with plasmids containing expI**. Each mutation was confirmed by sequencing, and plasmids were transformed by electroporation to an AHSL synthase-deficient (ExpI+) transposon mutant of E. carotovora without detectable AHSL production (SCC3065), which is derived from strain SCC3193 (15, 26).

**Determination of AHSL Content and Structure Elucidation**—AHLs were extracted twice with equal volumes of ethyl acetate from 2-ml culture supernatants grown in LB or minimal M9 medium (29) containing 0.4% sucrose or from 20 μl of macerated potato tuber tissue diluted in 500 μl of water. After evaporating to dryness in a SpeedVac, samples were dissolved in 20 μl (potato) or 200 μl (LB and M9) of 50% acetonitrile in 0.1% formic acid. 5 μl of the dissolved samples were injected for liquid chromatography-mass spectrometry analysis by an Agilent 1100 series high performance liquid chromatography system (Agilent Technologies) equipped with a Luna (Phenomenex) C18 column (100 μm × 4.6-mm inner diameter, 5-μm particle size) at a flow rate of 0.5 ml min−1 with 45 or 70% acetonitrile in 0.1% formic acid coupled without split to a quadrupole/time-of-flight mass spectrometer (Q-TOF Micro, Micromass Instruments). The positive electrospray ionization conditions included a capillary voltage of 3.2 kV, a cone voltage of 20 V, 5 eV ionization, a source temperature of 110 °C, and a desolvation temperature of 400 °C. Tender mass spectrometry spectra were generated with a collision energy of 12 eV with argon. AHL standards were purchased from Sigma-Aldrich (C7-, C8-, 3-oxo-C6-HSL) or synthesized (C4-, C6-, C9, 3-oxo-C8-HSL) as described (10). 3-OC-H6- and 3-OC-H8-HSL have been reduced from the corresponding 3-oxo-derivatives by stirring at room temperature with equimolar amounts of sodium borohydride in methanol for 15 min followed by purification over silica gel with chloroform/methanol. 3-OC-C5-, 3-oxo-C7-, 3-oxo-C9, 3-oxo-C10-., C5-, and C10-HSL have been identified by their high performance liquid chromatography retention time as well as by tandem mass spectrometry analysis.

**RESULTS**

**Specificity of AHSL Sensing in E. carotovora**—Most of the E. carotovora, Erwinia chrysanthemi, and P. stewartii strains characterized appear to produce and recognize the 3-oxo-C6-HSL autoinducer (1, 14, 12, 23, 31, 32). Accordingly, an AHL synthase-deficient transposon mutant (expI+) (SCC3065) (13, 26) of E. carotovora wild type strain SCC3193 exhibits strongly reduced virulence and extracellular enzyme production, which can be rescued by exogenous addition of this autoinducer (13).

However, the substrate specificity of the AHL synthases and the AHSL recognition specificity of the R-proteins is not absolute but may also include structurally similar AHSLs (15, 22). To assess the specificity of the autoinducer communication system in SCC3193, we compared the ability of AHSLs with different chain lengths and with or without a 3-oxo-group at the C5 position of the acyl chain to restore cellulase (Cel) production in SCC3065. For our experiments, <0.05 μM 3-oxo-C6-HSL is required to restore Cel production; this is >200 times less than the concentration of 3-oxo-C6-HSL needed to achieve the same effect (Fig. 1). The AHSLs with fully reduced acyl chains have intermediate activity with N-octanoyl-L-homoserine lactone (C8-HSL) as the most active compound (Fig. 1B).

**Immunoblot Analysis**—For Western blot hybridization, E. coli JM109 was grown overnight in triplicate, diluted into fresh LB medium, and grown until the A600 was 0.7. Expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside and, after 4 h, 1 ml of culture was collected. After an additional 2 h of growth, 2-ml supernatants of the same cultures were collected for AHSL measurement as described above. The pellet collected at 4 h was suspended in 100 μl of 6× PAGE sample buffer, and 10 μl were loaded to the gel. Protein extracts were separated on a 12.5% SDS-polyacrylamide gel and transferred with a buffer tank blotting system ( Hoefer, Amersham Biosciences) to nitrocellulose membrane (Nitro Bind, Micron Separations Inc.) using standard protocols (29). As the primary antibody, an anti-His6 monoclonal antibody (Qiagen) was used at a 1:2000 dilution according to manufacturer's instructions. Signals were visualized with rabbit anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (Pierce Biotechnology) and alkaline phosphatase substrate kit (Bio-Rad).

**Figure Preparation**—Homology modeling was performed with DeepView and SwissModel at swissmodel.expasy.org (30). Structure figures were prepared with Protein Explorer (molvis.sdc.edu/proteplxl/frndoor.htm), ISISDraw2.4, and Rasmol 2.5.

**SPECIFICITY OF AN ACYLHOMOSERINE LACTONE SYNTHASE**

![Fig. 1. Sensitivity of E. carotovora SCC3065 to AHSLs with different acyl side chains. A, SCC3065 was grown in LB with the indicated concentrations of 3-oxo-C6-HSL or 3-oxo-C8-HSL for 14 h. 10 μl of culture supernatant were applied to cellulose indicator media (carboxymethylcellulose) plates, incubated overnight, and developed with Congo Red to visualize Cel activity (13). B, minimal effective concentration (MEC) values of different AHSLs for inducing Cel activity as determined with carboxymethylcellulose plates.

This finding prompted us to characterize the AHSL profile of SCC3193 with liquid chromatography-mass spectrometry analysis (Fig. 2A and Table 1). Indeed, the main AHSL in SCC3193...
is 3-oxo-C8-HSL, and only traces of 3-oxo-C6-HSL can be detected. An inverse AHSL profile can be observed in another E. carotovora strain (SCC1) (27) with 3-oxo-C6-HSL as the main component. The type of AHSL produced is dependent on the corresponding I-protein (22, 23). C. potato tuber slices and the mean diameter of the macerated area (n = 10) 48 h post inoculation with 10⁶ colony-forming units of E. carotovora (n = 3). C. potato tuber slices and the mean diameter of the macerated area (n = 10) 48 h post inoculation with 10⁶ colony-forming units of E. carotovora. Bacterial strains and plasmids used in panels A, B, and C are indicated at the bottom of panel C. b. d., below detection limit; n. d., not detectable due to lack of maceration.

Interestingly, a series of AHSLs with unusual acyl chains having odd carbon numbers can be detected in bacterial cultures grown in rich medium (LB) but are undetectable in infected potato tissue or in cultures grown in a minimal medium (Table I and Supplemental Table I, available in the online version of this article). It seems that in the more stringent in planta environment only a few AHSLs have a role in determining virulence, as indicated by the poorer AHSL profile produced by E. carotovora in planta (Supplemental Table I). The specificity of SCC3193 for 3-oxo-C8-HSL in planta is also demonstrated by the inability of the 3-oxo-C6-HSL-producing ExpI3937 to complement the avirulent phenotype of SCC3065 in potato (Fig. 2C). This lack of complementation is not due to the inability of ExpI3937 to direct the synthesis of 3-oxo-C6-HSL in SCC3065 in planta as revealed by mixed infection with SCC3065 + expISCC1 and SCC3065 + expISCC193 (Fig. 2B). Taken together, our results demonstrate that the two different strains of E. carotovora use different dialects of the same AHSL language in their control of virulence and that these dialects are strain-specific at the physiological concentrations of the respective AHSLs.

Mutation Analysis Reveals Critical Residues for the Substrate Chain Length Specificity of the AHSL Synthase—The molecular basis for the distinct acyl chain length specificity of different autoinducer synthases, essential for the specificity of bacterial communication, has not been elucidated. The alignment of closely related ExpI-proteins with different chain length specificities augmented by the crystal structure of EsaI allows us now to identify candidates for the critical residues determining acyl chain length specificity. A comparison of the amino acid sequences of the relatively closely related I-proteins EsaI, CarI, ExpI3937, and ExpI3937 from E. chrysanthemi (32) (all producers of 3-oxo-C6-HSL) with ExpI3193 and ExpI3937 from another C8-producing E. carotovora with high homology to ExpI3193 revealed residues within close proximity of the putative acyl moiety-binding pocket (23) that are conserved in the C6 producers but different in the C8 producers (Fig. 3). These residues in ExpI3937 are Leu-67 (Val in EsaI and ExpI3937), Phe-69, Leu-123, and Met-127; the corresponding residues in ExpI3193 are Ser-67, Leu-69, Phe-123, and Thr-127 (numbering according to ExpI3193). The identity of residue 126 does not correlate with C6 or C8 production, as it is Ser-126 in CarI and ExpI3937, but Ala-126 in all other sequences. It was included in the subsequent studies because of its vicinity to the other residues of interest.

To elucidate the role of these residues in substrate chain length specificity, we changed each of them in ExpI3193 to the corresponding residue in ExpI3193. After introduction of each of the mutated ExpI3193 enzymes to E. coli JM109 and E. carotovora SCC3065, the resulting AHSL profile was analyzed. In SCC3065, the ability of the mutated enzymes to restore Cel production and virulence in potato was also investigated. The changes L67S (Fig. 4A, row 2) and F69L (Fig. 4A, row 3) reduced the amount of overall AHSL production in comparison to that of wild type ExpI3937 (Fig. 4A, row 1) but did not alter the type of AHSL produced. The modifications L123F and S126A (Fig. 4A, rows 4 and 5) had no effect on the amount or type of AHSL. In contrast, the change M127T (Fig. 4A, row 6) resulted in a drastic reduction of the overall AHSL content together with a clear shift to 3-oxo-C8-HSL production. Furthermore, this alteration enhanced Cel activity slightly and restored partly the ability of SCC3065 to infect potato tubers. A reverse change T127M in ExpI3193 background leads to a similar effect, decreasing overall AHSL content and causing a switch to 3-oxo-C6-HSL production (Fig. 4B, row 14), indicating that the identity of residue 127 is indeed important for ExpI substrate specificity.

Changing Specificity and Restoring Activity of the AHSL Synthase by Multiple Mutations—The low amounts of 3-oxo-C8-HSL produced by SCC3065 carrying the M127T mutated ExpI3193 suggested that additional changes in other residues of the putative substrate-binding pocket might be required. To test this hypothesis, we systematically changed each of the indicated amino acid residues in combination with the promising single change M127T (Fig. 4B, rows 1–10). This analysis revealed that the combinatorial change of F69L and M127T (Fig. 4B, row 3) both altered the acyl chain length specificity of ExpI3193 and restored AHSL production. The total amount of 3-oxo-C6-HSL produced by SCC3065 carrying this mutant ExpI corresponds to ~25% of that of the wild type ExpI3193. These changes completely restore Cel production in SCC3065.
and substantially enhance its virulence in potato. Further combinatorial changes appeared to improve and fine tune 3-oxo-C8-HSL production. The change S126A increases AHSL production to an ExpI variant directing the synthesis of even more 3-oxo-C8-HSL than wild type ExpISCC3193 (Fig. 4, rows 4, 9, 8, and 10), but a complete change of all five critical residues led to an ExpI variant directing the synthesis of even more 3-oxo-C8-HSL than wild type ExpISCC3193 (Fig. 4B, rows 10 and 12). The AHSL profiles produced in E. coli JM109 were similar to the profiles obtained in E. carotovora SCC3065 in all cases (data not shown).

The large differences in total AHSL content between various expI mutants could be due to differences in the copy number of plasmids or protein stability. To explore this possibility, we produced His6-tagged variants of ExpISCC1 wild type and the mutants M127T and F69L/M127T. The addition of a His6 tag and change of vector (pBS to pQE) did not appear to affect the AHSL production, but similar ratios of AHSLs were produced by both types of constructs (compare Figs. 5A and 4B, rows 1, 3, and 11). Immunoblot analysis showed a similar ExpI-protein content in all cases (Fig. 5B) and indicates that the difference in AHSL content (Fig. 5A) cannot be explained by differences in protein stability or plasmid copy number but rather reflects differences in ExpI activity.

**DISCUSSION**

In this study we describe the specificity of AHSL signaling in E. carotovora and demonstrate the molecular basis for the substrate chain length specificity of the AHSL synthase ExpI. We demonstrated that the E. carotovora strains SCC1 and SCC3193 produce 3-oxo-C6-HSL or 3-oxo-C8-HSL as their main autoinducer, respectively, and showed that the differences in chain length specificity of AHSL production is accompanied by a higher sensitivity toward 3-oxo-C8-HSL in the E. carotovora strain SCC3193 (Fig. 1). By mutation analysis we identified the residue 127 as critical for the determination of substrate chain length specificity of the AHSL synthase ExpI. The large differences in total AHSL content between various expI mutants could be due to differences in the copy number of plasmids or protein stability.

**FIG. 3.** Sequence comparison of I-proteins. Protein sequences are from E. carotovora (Ec) strains CFBP 6272 (GenBank™ accession number AJ580599), SCC3193 (X72891), SCC1 (AY507108), GS101 (X74299), E. chrysanthemi (Ech) strain 3937 (X96440), and Pantoea stewartii (Ps) strain SS104 (L32183). The I-proteins in the two uppermost lines produce mainly 3-oxo-C6-HSL (this study) (12, 31, 32, 23). Critical differences discussed in the text are marked with asterisks.

**TABLE 1**

Acyl-HSL production by E. carotovora SCC1 and SCC3193

| 14 h of growth in LB | 24 h p.i.\(^a\) in potato tubers |
|---------------------|-------------------------------|
| ExpI SCC1 | ExpI SCC3193 | ExpI SCC1 | ExpI SCC3193 |
| 3-Oxo-C5-HSL | 16.7 ± 7.6 | BD\(^b\) | 2028.7 ± 406.4 | BD\(^b\) |
| C5-HSL | 3.8 ± 2.0 | BD\(^b\) | 177.6 ± 12.7 | BD\(^b\) |
| 3-Oxo-C6-HSL | 86.4 ± 109.8 | 18.9 ± 1.0 | 75.1 ± 65.1 |
| C6-HSL | 51.0 ± 4.0 | 3.5 ± 0.8 | 94.7 ± 6.0 | BD\(^b\) |
| 3-Oxo-C7-HSL | 70.1 ± 33.5 | 23.3 ± 9.8 | BD\(^b\) |
| C7-HSL | 7.8 ± 2.9 | 18.6 ± 10.7 | BD\(^b\) |
| 3-Oxo-C8-HSL | 3.5 ± 1.0 | 679.2 ± 22.0 | 7.9 ± 6.8 | 169.1 ± 31.9 |
| C8-HSL | BD\(^b\) | 94.7 ± 6.0 | BD\(^b\) |
| 3-OH-C8-HSL | BD\(^b\) | 6.2 ± 0.9 | BD\(^b\) |
| C9-HSL | BD\(^b\) | 15.1 ± 4.2 | BD\(^b\) |
| 3-Oxo-C9-HSL | BD\(^b\) | 21.3 ± 13.0 | BD\(^b\) |
| C10-HSL | BD\(^b\) | 9.3 ± 0.6 | BD\(^b\) |
| 3-Oxo-C10-HSL | BD\(^b\) | 0.5 ± 0.2 | BD\(^b\) |

\(^a\) Post inoculation with 10\(^6\) colony forming units.

\(^b\) Ec, E. carotovora.

\(^c\) Below detection limit.

\(\text{nm} \pm \text{S.E.}\)
of the substrate chain length by the AHSL synthase ExpI in E. carotovora. The additional change F69L led to the recovery of enzyme activity, and additional multiple amino acid changes restored production of the changed product 3-oxo-C8-HSL to wild type levels.

This high specificity of AHSL signaling raises the question of why closely related strains of E. carotovora use different signal molecules. An effective change of specificity required at least two independent mutations in the I-protein alone (Fig. 4). Together with the specificity in the sensing mechanism, it seems unlikely that the specific use of 3-oxo-C6 or 3-oxo-C8 signaling is pure coincidence but might reflect a biological or ecological difference in function. The important role quorum sensing plays in virulence could reflect a role in the interplay of the bacteria with its eucaryotic hosts. Indeed, several studies indicate that eucaryotic organisms, including plants, are able to respond to bacterial AHSL signals (see Ref. 34). A possible explanation for the use of distinct AHSLs might reflect differential efficacy of a specific AHSL in different host varieties or tissues. The host might recognize only certain AHSLs, or specific AHSLs may have different diffusion abilities in the host milieu, thereby influencing the required signaling capacities. Alternatively, differentiation in AHSL signaling could be a result of competition between bacterial strains.

Mutation analysis and the introduction of plasmids encoding different I-proteins to the AHSL negative strain SCC3065 has demonstrated that the composition of the AHSL profile is mainly dependent on the I-protein introduced. However, this profile is modulated by external milieu. Different growth media or different growth conditions result in either very complex AHSL profiles containing >10 different AHSLs (in LB) or in profiles containing a few AHSLs only (in M9 minimal medium and in potato; Table I and Supplemental Tables I and II, available in the on-line version of this article). These data suggest that although the I-protein determines the type of AHSL produced, fine tuning of the AHSL composition can be achieved in different growth conditions. The rich media itself does not seem to directly provide fatty acids with odd acyl chain length as in AHSL precursors, because the addition of LB medium to macerating potatoes did not result in increased values of AHSLs with odd chain lengths (data not shown).

Rather, bacterial metabolism appears to be regulated differently in growth conditions in LB medium, thereby resulting in the production of larger amounts of these unusual AHSLs. Here, the availability of precursors with different acyl chain length in the acyl-ACP pool might play an important role. In P. aeruginosa, a series of fatty acid biosynthesis (fab) genes are responsible for the production of acyl-ACPs and, consequently, are required for the production of AHSLs (35). Differential regulation of acyl-ACP synthesis as a consequence of altered growth conditions could also explain the differences in AHSL profiles in E. carotovora. Acyl-ACPs with odd chain length might arise via a pathway that uses propionyl-CoA instead of malonyl-CoA or acetyl-CoA as the respective chain starter (36, 37). Previously, only a few AHSLs with odd chain length have been described; Rhizobium leguminosarum produces heptanoyl-HSL (C7-HSL) (38), and here also the extent of C7-HSL accumulation is higher in rich medium than in minimal medium (39). An alternative possibility might be that under specific growth conditions (in potato or in minimal medium) uncharacterized E. carotovora enzymes more efficiently degrade AHSLs and/or acyl-ACPs with a particular chain length.
determined by immunoblot with anti-His5 antiserum of the cultures of mutants from cultures of expI
A from the same growth as in /H9252

Comparison of AHSL production with I-protein content. A, 3-oxo-C6-HSL and 3-oxo-C8-HSL content (n = 3) in supernatants from cultures of E. coli JM109 (in LB) carrying expI SCC1 genes on pQE plasmids with an N-terminal His6-tag 6 h after the addition of isopropyl /D-thiogalactopyranoside. B, I-protein content of cell lysates determined by immunoblot with anti-His6 antiserum of the cultures from the same growth as in panel A 4 h after the addition of isopropyl /D-thiogalactopyranoside. The marker on the left indicates the molecular mass in kDa. Amino acid changes are indicated. b, d.: below detection limit.

Our results demonstrate that the change M127T is critical for the shift in substrate specificity of the AHSL synthesizing protein ExpI SCC1. The other changes, particularly F69L and S126A, recover wild type levels of activity of the enzyme. One possible explanation for these observations is that the changes affect protein stability or expression of the expI variants on plasmids. This seems not to be the case, because immunoblot analysis shows that protein levels of His6-tagged ExpI SCC1 wild type and the representative mutants M127T and F69L/M127T are similar despite drastic differences in the production of AHSLs (Fig. 5A). Another indication that protein stability is not affected is that the mutations do not only change the total amount of AHSL produced but also change the ratio between various types of AHSLs (Supplemental Table II).

A more likely explanation is that the mutations change the structure of the ExpI protein, especially in the region of a putative acyl moiety-binding pocket. It seems that M127T increases the size of the catalytic pocket to specifically fit in 3-oxo-C8-ACP, and the changes F69L and S126A cooperate in optimizing the pocket size (Fig. 4B, rows 1, 3, 5, and 7). How can this be achieved? The change of Met-127 to the more hydrophilic amino acid Thr is likely to alter the interactions with the adjacent Phe-124 or with the water molecules surrounding the hydrophobic cavity site. This leads to a conformation of residue127 accompanied by a change in the size of the putative acyl (here C6)-binding pocket. The effect of the mutation F69L can be explained by the size difference between these residues. Indeed, homology modeling (30) using the EsaI crystal structure as a template shows that Phe-69 and Met-127 are in close contact in ExpI SCC1 and seem to form the end of the C6-binding pocket. The mutations M127T and F69L widen the gap between the two residues, thereby creating space for the larger C8 side chain (Fig. 6). The hydrophilic Ser-126 seems to interfere with Thr-127, therefore, a change to the more hydrophobic and smaller amino acid Ala enhances the effect of Thr-127 on the acyl-binding pocket size.

The effect of the mutation L67S is more complex and more difficult to explain. Although the mutation has a negative effect on yield, in combination with multiple other mutations this negative influence disappears and might even result in enhanced AHSL production (Fig. 4B, row 7 compared with row 8). Possibly, after rearrangement of the catalytic pocket region to an effective C8-producer by the mutations F69L, S126A, and M127T, the additional mutation L67S can result in a positive effect via an unknown interaction with the more hydrophilic amino acid Ser.

Multiple mutations of ExpI SCC1 not only lead to an increase in production of 3-oxo-C8-HSL but also to a decrease in production of 3-oxo-C6-HSL (Fig. 4 and Supplemental Table II). Why is the increased catalytic pocket not functional for the smaller ligand? The proposed N-acylation mechanism of the respective acyl-ACP precursor with S-adenosyl-l-methionine might be of importance in this respect (23). After proton abstraction from the protonated amine group of S-adenosyl-l-methionine catalyzed by Glu-98 and/or Ser-100, the free electrons of the amine group can react via a nucleophilic attack with the 1-carbonyl of the respective acyl-ACP, and the resulting negatively charged anion intermediate could be stabilized by Arg-101 and Phe-102 (23). These amino acids are conserved between several I-proteins, and mutations at these residues often lead to non-functional I-proteins (1, 4, 23). Widening of the hydrophobic cavity as shown in Fig. 6 could shift the relative position of the 1-carbonyl group of the C6-ACP in relation to those critical amino acids and therefore reduce the overall effectiveness of the catalytic process with C6-substrates.

Interestingly, the mutant forms of ExpI SCC1 have a significantly higher activity toward C8-HSL than ExpI SCC1 in a specific reaction (Supplemental Table II), e.g. the triple mutant F69L/S126A/M127T produces even more C8-HSL than C6-AHSL. This suggests that in addition to the earlier identified Thr-141 (Thr-140 in EsaI) (23), other residues are also determining the specificity to substrates.
3-oxo-HSL. It is also possible that additional residues not necessarily in close proximity to the acyl-ACP-binding pocket might influence the ratio of AHSLS produced. In this context it is interesting to note that the amino acids 209 and 210 also correlate with C6 and C8 specificity (Fig. 3) and might play a role in further fine tuning of I-protein specificity.

Disruption of bacterial communication systems is a promising avenue for the control of bacterial pathogens both in plant and animal diseases (2, 5, 40). Different approaches to achieve this include the use of bacterial enzymes to degrade autoinducers (41, 42), overexpressing AHSLS in planta (43, 44) or the development of drugs inhibiting autoinducer signaling (40). Understanding and exploitation of the molecular basis of substrate chain length specificity might allow further development of novel tools for the quorum sensing-based control of pathogenic Gram-negative bacteria.

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