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Proferrioxamine synthesis in *Erwinia amylovora* in response to precursor or hydroxylysine feeding: metabolic profiling with liquid chromatography–electrospray mass spectrometry

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Metabolic profiling by capillary liquid chromatography–electrospray mass spectrometry was used to monitor shifts in the proferrioxamine profiles of *Erwinia amylovora* in response to externally supplied potential proferrioxamine precursors, selected stable-isotope-labeled precursors and atypical precursors. Based on the qualitative and quantitative shifts in the proferrioxamine profiles, lysine and arginine are unambiguous, and agmatine, ornithine, diaminobutyric acid and the corresponding C_{3-5} diamines are highly likely precursors for proferrioxamine biosynthesis in *E. amylovora*. 5-Hydroxylysine (Hyl), a recently discovered growth inhibitor for *E. amylovora*, suppresses proferrioxamine production. The Hyl-induced growth inhibition can be reversed by basic amino acids. The basic amino acids also partly restore proferrioxamine synthesis.

Keywords: siderophores, proferrioxamine biosynthesis, *Erwinia amylovora*, fire blight, *Enterobacteriaceae*, antibiotic resistance, metabolic profiling, precursor feeding, growth inhibition, 5-hydroxylysine, mass spectrometry, stable-isotope labeling

Introduction

Erwinia amylovora is the etiological agent of fire blight, an often devastating disease of rosaceous plants, especially of pome trees. Development of resistance to streptomycin, the most widely used control agent for fire blight, has prompted the search for alternative control agents, either chemical (Zeller 1992) or biological agents (Naumann & Gierz 1992, Johnson *et al.* 1993), but thus far the search is largely by trial and error. The development of new fire blight control agents would be aided if specific vulnerable sites in *E. amylovora* were known. One potentially useful target may be proferrioxamine (pFO) synthesis in *E. amylovora*, since many (Ankenbauer & Cox 1988, Enard *et al.* 1988, Visca *et al.* 1992, Griffiths 1993, Neema *et al.* 1993, Boelaert *et al.* 1994, Chambers & Sokol 1994), although not all (Leong & Neilands 1981, Ishimaru & Loper 1992, Griffiths 1993, Lambrecht & Collins 1993, Mei *et al.* 1993), plant and animal pathogens depend on siderophores to acquire essential iron from and to establish themselves in their hosts. With regard to the chemical structures and the nomenclature of proferrioxamines, the reader is referred to my earlier communication (Feistner 1995a).

Previous attempts to demonstrate a correlation between proferrioxamines and virulence in *E. amylovora* with pFO^- mutants have been inconclusive (Sneath *et al.* 1990, Vanneste & Expert 1990, Vanneste *et al.* 1990, Feistner *et al.* 1993a), in part because of the likelihood of alternative biosynthetic pathways for proferrioxamines, and in part because a phenotypical lack of siderophore excretion is not necessarily due to a defect in a stuctural gene but may rather be due to a defect in a regulatory gene (Feistner *et al.* 1993b). Complementary biochemical investigations are needed to overcome these obstacles. I have therefore embarked on a systematic analysis of proferrioxamine synthesis in *E. amylovora*, taking advantage of the speed and specificity of metabolic profiling by on-line liquid chromatography–electrospray mass spectrometry (Feistner 1994b). I already reported on some aspects of proferrioxamine biosynthesis in *E. amylovora*, i.e. the metabolism of polyamines and basic amino acids (Feistner 1994d). It was, however, not immediately clear from

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Part 12 in the series 'Metabolites of *Erwinia*', for Parts 10 and 11 see Feistner (1994d) and Feistner (1995b), respectively. Presented, in part, at ALEX '93, San Francisco, October 5–7, 1993, and at the 42nd ASMS Conference, Chicago, May 29–June 3, 1994.

my previous study whether these putative precursors would actually be incorporated into proferrioxamines. Detection of the precursors and their immediate metabolic products required dansylation, which unfortunately destroys proferrioxamines. Hence the same culture supernatants were also analyzed without derivatization, using a profiling method previously described by us (Feistner *et al.* 1993b). The results of these additional studies, which are shown below, prove that basic amino acids and diamines are indeed proferrioxamine precursors, whereas polyamines probably are not. In addition, I provide evidence that 5-hydroxylysine (Hyl), a serendipitously discovered growth inhibitor for *E. amylovora* (Feistner 1994a,d), reduces proferrioxamine production.

The present study was carried out in two stages (first with unlabeled, then with labeled or atypical precursors) for the following reasons. Unambiguous demonstration of the incorporation of putative precursors into proferrioxamines requires labeled precursors. In the case of mass spectrometric studies such as described here, labeling with stable isotopes of hydrogen, carbon or nitrogen is appropriate. Unfortunately, the cost for a comprehensive labeling study with all possible proferrioxamine precursors was prohibitively high, since most of the labeled precursors are not available from the shelf and have to be custom synthesized. I therefore first conducted directed fermentation studies with all putative precursors in their unlabeled form. I expected that true precursors would identify themselves by overstimulating the production of a corresponding subset of proferrioxamines; an assumption that was based on previous directed fermentation studies in streptomyces (Meiwes *et al.* 1990). The extension of this method to *E. amylovora* proved to be feasible. Subsequent selected feeding experiments with atypical and labeled precursors allowed to distinguish biosynthetic from regulatory effects.

Materials and methods

Chemicals

Most precursors were obtained as hydrochloride salts from Sigma (St Louis, MO). Diaminopropane, diaminohexane and diaminoheptane were obtained from Aldrich (Milwaukee, WI) and neutralized with 10% sulfuric acid [our previous inability (Feistner *et al.* 1993b) to grow *E. amylovora* in the presence of these diamines may have been due to inadvertent omission of this neutralization step]. [$^{15}N_2$]-L-lysine hydrochloride (≥95% labeled) and [$^{13}C_6$]-L-arginine hydrochloride (≥98% labeled) were obtained from Cambridge Isotope Laboratories (Woburn, MA). The synthesis of 2-hydroxyputrescine is described elsewhere (Feistner 1994c).

Culturing of E. amylovora

EA1430 was exclusively used for this study and was grown as described (Feistner 1994d). The analysis of the spent culture supernatants for basic amino acids and polyamines suggests that these precursors are not used as carbon sources by *E. amylovora*, at least not in the presence of sucrose, since large amounts of the labeled and unlabeled amino acids and their decarboxylation products were detected (Feistner 1994d).

Preparation of extracts

Methanolic extracts of the dried culture supernatants were prepared as described (Feistner 1994d). The final extracts were 1 ml aqueous solutions, except for the methionine feeding study where the corresponding extract had to be dissolved in 2 ml because of the poor solubility of methionine. The quantitative studies with lysine and cadaverine included pFO_{555Ac} as an internal standard. pFO_{555Ac} (16 μg) was added to 25 ml culture supernatant, then the extracts were prepared as above.

Identification of proferrioxamines

Extracts were analyzed by capillary reversed-phase liquid chromatography–electrospray mass spectrometry (LC-ES-MS) using the City-of-Hope microgradient system (Feistner *et al.* 1993b) and a Finnigan (San Jose, CA) TSQ700 triple stage quadrupole mass analyzer fitted with either an Analytica (Branford, CT) or a Finnigan electrospray source. Individual proferrioxamines were identified based on their respective molecular ions and relative retention times as described (Feistner *et al.* 1993b). The injection volume typically was 1 μl, but in the quantitative studies with lysine and cadaverine the injection was preceded by a 1:10 dilution. A linear gradient of 1–91% acetonitrile in 0.1–0.07% aqueous trifluoroacetic acid, delivered over 1–60 min at 2 μl min^{-1}, was employed throughout. A liquid sheath of 2 μl min^{-1} 2-methoxyethanol and nebulization with N_2 (60 psi; 414 kPa) were used to facilitate the electrospray process. To avoid contamination of the transfer capillary between electrospray source and mass analyzer with salts and sugar, the electrospray ionization was started only after the void volume had passed. Fast solvent blank runs (20 μl min^{-1}) were performed between individual samples to eliminate cross-contamination. Differentiation between the isobaric pFO_{445c} and pFO_{355c} was primarily based on the relative elution order with regard to pFO_{555}. In our C_{18} reversed phase capillary chromatographic system, pFO_{445c} elutes before and pFO_{355c} after pFO_{555}. In the case of the diaminobutyric acid-fed culture, the assignment of pFO_{355c} was also corroborated by tandem mass spectrometry (major fragment ions at *m/z* 173, 201, 373 and 401) as described previously (Feistner *et al.* 1993b).

Quantitative estimates

To determine the approximate concentrations of proferrioxamines, the corresponding LC peak areas were normalized to the internal standard pFO_{555Ac}. Making the assumptions that the mass spectrometric response below 10^{-5} M is roughly a linear function of compound concentration [an assumption that is supported by the literature (Ikonomou

et al. 1990, Hopfgartner *et al.* 1993, Tang & Kebarle 1993) and also consistent with the simultaneously recorded UV chromatograms], and that there are no significant differences in the response for different proferrioxamines, the calculated peak ratio (pFO_X/pFO_{555Ac}) was multiplied by a factor of 640 (16 $\mu g/25$ ml = 640 $\mu g\,l^{-1}$) to obtain the concentration of pFO_X in $\mu g\,l^{-1}$.

Profiling for 6-thioguanine

In several instances of delayed growth, it was important to make sure that the growth was indeed due to *E. amylovora* and not some air-borne bacterium. One way to largely rule out such contamination is by demonstrating the presence of typical metabolites for *E. amylovora*. Often, the proferrioxamines could serve this purpose; however, in the iron-supplementation studies this was not feasible, because iron at $>10^{-6}$ M suppresses proferrioxamine synthesis (Feistner *et al.* 1993a). In these instances, the extracts were analyzed for 6-thioguanine (6-TG), another metabolic marker for *E. amylovora* (Feistner & Staub 1986). Since no LC-ESI-MS profiling method for 6-TG is available yet (Feistner 1994b) conventional reversed-phase HPLC analysis (C_{18}; 2.1 × 220 mm; plus 30 mm precolumn; Applied Biosystems, Foster City, CA) with absorbance detection at 340 nm was used. The analyses were performed on a Hewlett-Packard (Palo Alto, CA) 1090M workstation, using a linear gradient of 0–50% B over 1–50 min (A = 100% water; B = 90% methanol), a flow rate of 0.2 ml min^{-1}, peak controlled acquisition of absorbance spectra (210–400 nm) and an injection volume of 50 μl. 6-TG was identified on grounds of its retention time of approximately 13.8 min and its characteristic UV absorbance spectrum (Feistner & Staub 1986).

Results and discussion

The proferrioxamine profiles that were obtained after feeding 12 unlabeled, putative precursors are shown in Figure 1 (only the relevant portions of the respective mass chromatograms are shown). Due to chemical background noise in the mass spectrometric measurement, on one hand, and facile saturation of the electrospray ionization process (at approximately 10^{-5} M pFO) and the low sample capacity of the capillary column, on the other, the dynamic range for the measurements was limited to two to three orders of magnitude. To be able to detect the less abundant proferrioxamines, the major ones were frequently overloaded. Thus, the abundance of specific proferrioxamines has to be judged not only from the peak heights but also from the peak widths. To evaluate the profiles with regard to precursor incorporation, only the relative proferrioxamine abundances within a given profile (a column in Figure 1) need to be considered. Differences in absolute abundancies between the different feeding studies are also seen but these are not significant because measurements were done over an extended period of time under varying mass spectrometric conditions.

The easiest way to evaluate the profiles is to divide the proferrioxamines of *E. amylovora* into three groups (indicated on the right hand side of Figure 1). Group 1 consists of those proferrioxamines that only contain cadaverine (pFO_{555c}, pFO_{555} and pFO_{5555c}). Group 2 consists of proferrioxamines, which, in addition to cadaverine, contain putrescine but no diaminopropane (pFO_{455c}, pFO_{4555c}, $pFO_{(455)}$, pFO_{4455c}, $pFO_{(4455)c}$, $pFO_{(445)}$ and pFO_{444c}). Finally, group 3 consists of those proferrioxamines that contain diaminopropane (pFO_{355c}, $pFO_{(345)c}$ and pFO_{3555c}). Under basic culture conditions (plain MMB medium), *E. amylovora* produces most of the above in various but fairly reproducible amounts. Following the feeding of any putative precursor, the observation of a predictable shift in the relative abundance between the three proferrioxamine groups can be taken as a strong indication for the incorporation of this precursor. Thus, incorporation of cadaverine, putrescine and diaminopropane (or appropriate precursors thereof) should lead to a relative increase in group 1, 2 and 3 proferrioxamines, respectively. Two especially tell-tale signs of the proferrioxamine profiles are the relative ratios of pFO_{455c} and pFO_{555c} (for the purpose of comparing the degree of putrescine versus cadaverine incorporation) and of the isobaric pFO_{445c} and pFO_{355c} (for the purpose of comparing the degree of putrescine versus diaminopropane incorporation). Without added precursors, these ratios are roughly 1:1, and the variability between batches is typically not larger than a factor of 2.

Feeding of polyamines and methionine

The relative molecular mass profiles observed after feeding spermidine or spermine were found to be very similar to those obtained under basic culture conditions (compare Figure 2 in Feistner *et al.* 1993b). Indeed, the similarity is such that the spermine- and spermidine-related profiles will be used as reference for the discussion of Figure 1. Normal 1:1 ratios for pFO_{455c}:pFO_{555c} and pFO_{445c}:pFO_{355c} were observed. The same holds true for the profiles observed after feeding methionine or either one of the two isomeric acetylspermidines. These observations are consistent with my previously reported finding (Feistner 1994d) that spermidine and its acetyl derivatives are not appreciably degraded by *E. amylovora* and that in the case of spermine acetylated rather than free diamines are the final degradation products. *Escherichia coli*, a closely related bacterium, also does not degrade spermidine (Tabor & Dobbs 1970), whereas some bacteria outside the family *Enterobacteriaceae* are able to do so (Razin *et al.* 1959, Bachrach *et al.* 1960, Bachrach 1962). Based on these combined results, stable-isotope-labeling studies for methionine and polyamines were not pursued.

Feeding of lysine and cadaverine

The most striking change in the proferrioxamine profiles was observed after feeding lysine. Strongly elevated levels of group 1 proferrioxamines contrasted with the nearly

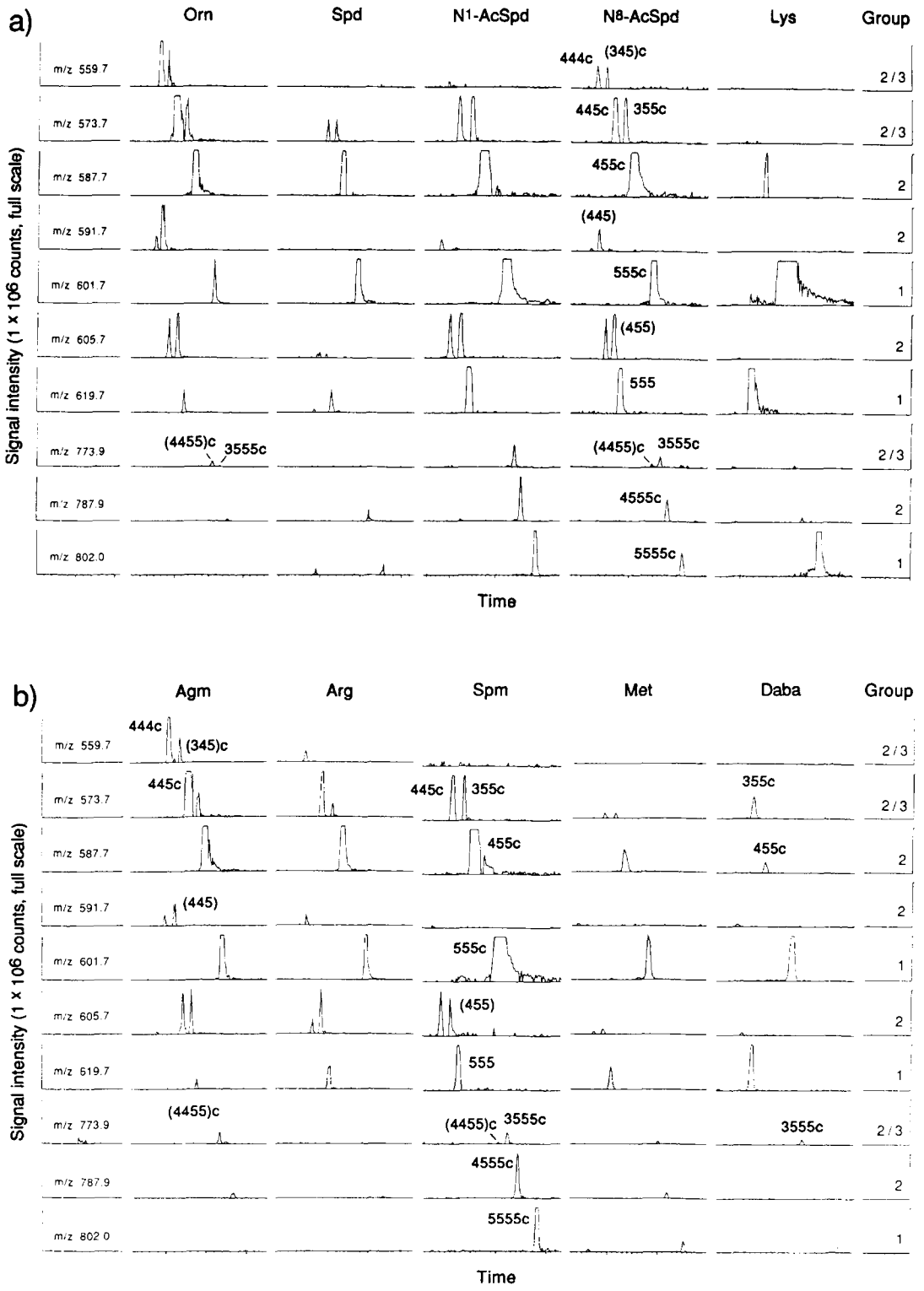

Figure 1. Proferrioxamine profiles of *E. amylovora* in response to precursor feeding. Each column represents a separate feeding experiment: the corresponding precursors are indicated at the top of the figure. Shown are the relative abundances of the various proferrioxamines as revealed by mass chromatography. *m/z* numbers on the left hand side of the figure indicate the corresponding protonated molecular masses, while numbers on the right hand side indicate the corresponding proferrioxamine group (see text). '2/3' refers to the presence of isobaric proferrioxamines belonging to group 2 and 3, respectively. To avoid cluttering, only one column each in (**a**) and (**b**) has been fully labeled.

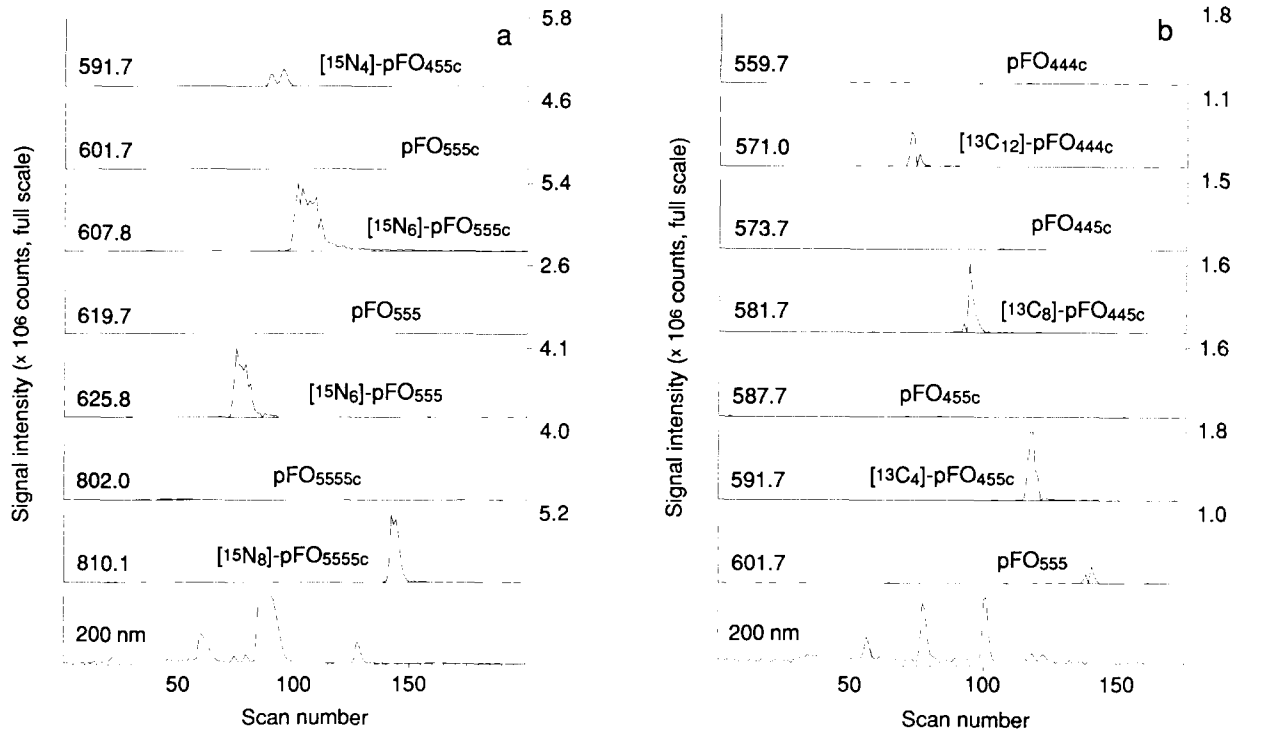

Figure 2. Incorporation of stable-isotope-labeled Arg and Lys. (**a**) [$^{15}N_2$]lysine study and (**b**) [$^{13}C_6$]arginine study. Incorporation of the label was evaluated by monitoring for predictable mass shifts in the most abundant proferrioxamines under each feeding condition (see Figure 1 and Results and discussion). Absorbance chromatograms are shown in the bottom panels. The obvious time delay between the absorbance and mass spectrometric profiles is due to the fact that absorbance and mass spectrometric detection were in series (absorbance detection first).

complete absence of all other proferrioxamines. In this particular study, the impact of lysine on proferrioxamine production was even more dramatic than the previously observed effect of cadaverine (Feistner *et al.* 1993b). This was quite unexpected since it was in stark contrast to a previous report for pFO_{555c} synthesis in *Streptomyces glaucescens*, where a 3-fold higher incorporation of [1,5-^{14}C]cadaverine over [4,5-^{3}H]lysine was demonstrated (Schafft & Diekmann 1978); this unexpected finding prompted additional differential profiling studies that are discussed below.

Feeding of putrescine precursors

Ornithine led to dramatic increases in the relative abundance of group 2 proferrioxamines. Thus, pFO_{445c} and pFO_{444c} are much more abundant than pFO_{355c} and $pFO_{(345)c}$, respectively, and the amount of pFO_{455c} is much larger than that of pFO_{555c}. Similar relative increases for pFO_{455c}, pFO_{445c} and pFO_{444c} were also observed after feeding agmatine and arginine. It is interesting to note that while the proferrioxamine profile shifts induced by ornithine and arginine were in the same ballpark, the biosynthetic intermediate putrescine accumulated to high levels in the growth medium only in the feeding study with ornithine (Feistner 1994d).

Feeding of diaminobutyric acid and diaminopropane

Diaminobutyric acid was found to augment the biosynthesis of group 3 proferrioxamines while suppressing the biosynthesis of group 2 proferrioxamines. For example, a strong pFO_{355c} peak is observed but virtually none for pFO_{445c}, and the pFO_{555c} peak is much stronger than the one for pFO_{455c}; the latter being even smaller than the peak for pFO_{355c}. Also, pFO_{3555c} is of about equal abundance as pFO_{5555c}, while hardly any pFO_{4555c} is observed. The proferrioxamine profile obtained for the diaminopropane-complemented culture was very similar (data not shown). In addition, a small peak with a protonated molecular mass of 545 was detected which, since it was not observed in any of the other profiles, most likely represented pFO_{335c}.

Feeding of [$^{15}N_2$]-L-lysine and [$^{13}C_6$]-L-arginine

The above data indicated that all basic amino acids are precursors for proferrioxamine biosynthesis in *E. amylovora*. For two of the four basic amino acids, i.e. lysine and arginine, this was subsequently proven with stable-isotope-labeled compounds. As expected, multiples of [$^{15}N_2$]cadaverine and [$^{13}C_4$]putrescine were incorporated (Figure 2). Thus, incorporation of two, three and four [$^{15}N_2$]cadaverine moieties led to a mass shift of 4 u in pFO_{455c}, of 6 u in

pFO_{555c} and pFO_{555}, and of 8 u in pFO_{5555c}, while incorporation of three, two and one [$^{13}C_4$]putrescine moieties led to mass shifts of 12, 8 and 4 u in pFO_{444c}, pFO_{445c} and pFO_{455c}, respectively. In both labeling studies, the labeled pFOs were the most abundant metabolites, whereas the corresponding unlabeled pFOs were undetectable. This proves the ready incorporation of lysine and arginine into pFOs in *E. amylovora*.

Feeding of diaminohexane and 2-hydroxyputrescine

Because it is conceivable that the basic amino acids may be incorporated into proferrioxamines via pathways that do not involve the corresponding free diamines (see below), feeding fermentations with atypical diamines, i.e. diaminohexane, diaminoheptane and 2-hydroxyputrescine, were initiated. As could be expected from the results of equivalent studies in streptomyces (Meives *et al.* 1990), diaminohexane, but not diaminoheptane, was incorporated into proferrioxamines. Figure 3(a) demonstrates the presence of pFO_{556c} and $pFO_{(556)}$, thus indicating the formal replacement of one cadaverine moiety each in pFO_{555c} and pFO_{555} by diaminohexane. These new proferrioxamines are apparently synthesized at the expense of pFO_{455c}. The feeding of 2-hydroxyputrescine also led to a new proferrioxamine, i.e. HO-pFO_{455c} [in analogy to alcaligin (Feistner 1995a) probably 8-HO-pFO_{455c}] (Figure 3b), in which the putrescine moiety in pFO_{455c} is replaced with hydroxyputrescine. As expected, $HOpFO_{455c}$ was more hydrophilic than pFO_{455c}, a characteristic that is also shown between the corresponding ferric complexes (HO-pFO_{455c} and pFO_{455c}).

pFO_{556c} and HO-pFO_{455c} were both confirmed by tandem mass spectrometry (Figure 4). We have previously shown that cyclic proferrioxamines give characteristic fragment ion spectra that readily reveal the number and sizes of the constituting diamines (Feistner *et al.* 1993b). For example, pFO_{555c} gives rise to abundant ions with *m/z* 201 and 401. For the homologous pFO_{556c} one would thus expect to see additional fragments at *m/z* 215 and 415, and this was indeed observed (Figure 4a). pFO_{455c} has been shown to yield abundant fragment ions with *m/z* 187, 201, 387 and 401, where the *m/z* 187 and 387 ions, but not the *m/z* 201 and 401 ions, contain putrescine (Feistner *et al.* 1993b). Thus, HO-pFO_{455c} is expected to give rise to a mass shift of 16 u for *m/z* 187 and 387, resulting in new ions with *m/z* 203 and 403. Again, that was observed (Figure 4b). These data prove that diamines are bona fide biosynthetic intermediates between basic amino acids and proferrioxamines [the corresponding decarboxylase activities had been demonstrated in *E. amylovora* earlier (Feistner 1994d)] and not just regulatory activators of proferrioxamine biosynthesis in *E. amylovora*.

Differential profiling following feeding with lysine or cadaverine

The proof that diamines are true intermediates of proferrioxamine biosynthesis does not exclude other, parallel biosynthetic pathways that involve primary

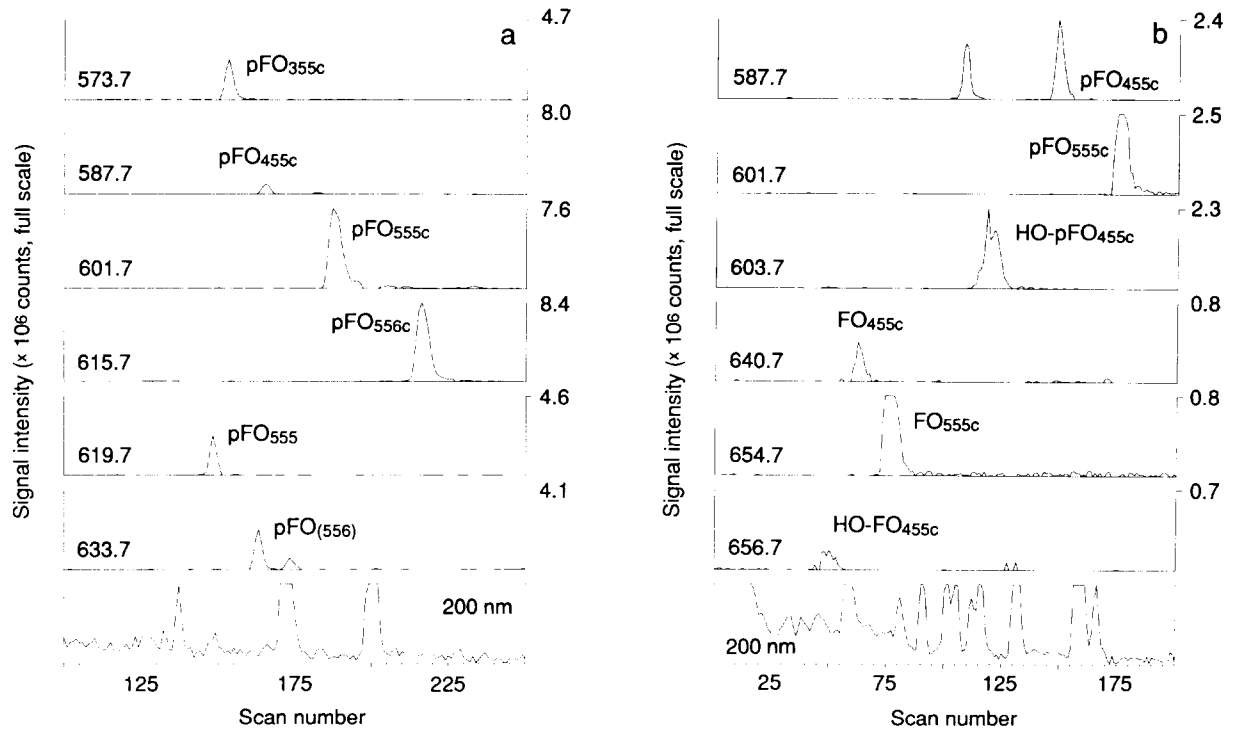

Figure 3. Incorporation of atypical precursors. Incorporation of (**a**) diaminohexane and (**b**) 2-hydroxyputrescine. Shown are the absorbance chromatograms (bottom panel) and (delayed) the mass chromatographic profiles for selected proferrioxamines. The metabolic profile in (**b**) is obviously very complex and has not yet been fully analyzed, but this only demonstrates the power of mass chromatographic searches.

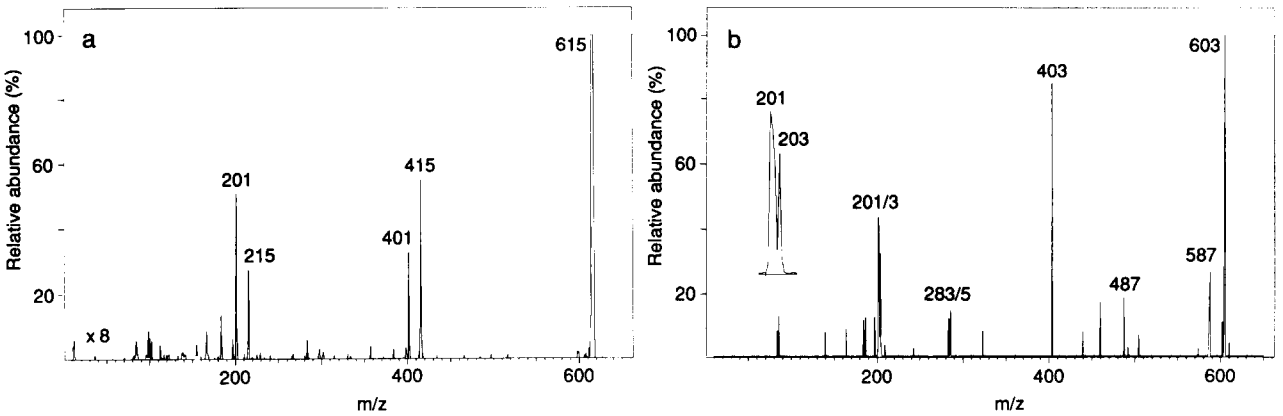

Figure 4. Structure confirmation of atypical pFOs by tandem mass spectrometry. Tandem mass spectra of (**a**) pFO_{556c} and (**b**) HO-pFO_{455c}. The spectra were obtained by averaging seven and four scans, respectively, over the corresponding LC peaks, and are consistent with what was predicted based on previous tandem mass spectrometric studies of a large number of proferrioxamines (Feistner *et al.* 1993b).

N-hydroxylation and/or succinylation of basic amino acids (Figure 5). Indeed, the more dramatic proferrioxamine profile shift caused by lysine versus that previously seen with cadaverine (see above) suggested that such other pathways may be active. However, different strains of *E. amylovora* had been used in the current lysine and the previous cadaverine feeding studies. The differential analysis therefore needed to be repeated with a single strain under identical conditions before any conclusion could be drawn. The repeat study included pFO_{555Ac} as internal standard. To bring the proferrioxamine concentration down to the low micromolar range, where the ESI-MS response could be expected to be a linear function of the proferrioxamine concentration (Hopfgartner *et al.* 1993, Tang & Kebarle 1993), the extracts were diluted 1:10 before measurement. Even then, saturation of the main pFO_{555c} signal was evident. In order not to loose the information for minor proferrioxamines, no further dilution was made. Instead, pFO_{555} and pFO_{5555c} were chosen to represent group 1 proferrioxamines. The signal intensities for the latter and pFO_{455c} were of the same order as the signal intensity for the internal standard pFO_{555Ac}, and this allowed a meaningful comparison even without exact quantification. Other than expected, the concentrations of pFO_{555} and pFO_{5555c} in the Lys- and the Cad-supplemented cultures were basically the same (Figure 6). There was a reproducible difference in the proferrioxamine profiles between the Lys- and Cad-supplemented cultures but it related to group 2 rather than group 1 proferrioxamines. Lysine significantly suppressed pFO_{455c} production, whereas cadaverine did so to a much smaller extent. The data did not support the expectation that cadaverine is preferentially incorporated over lysine.

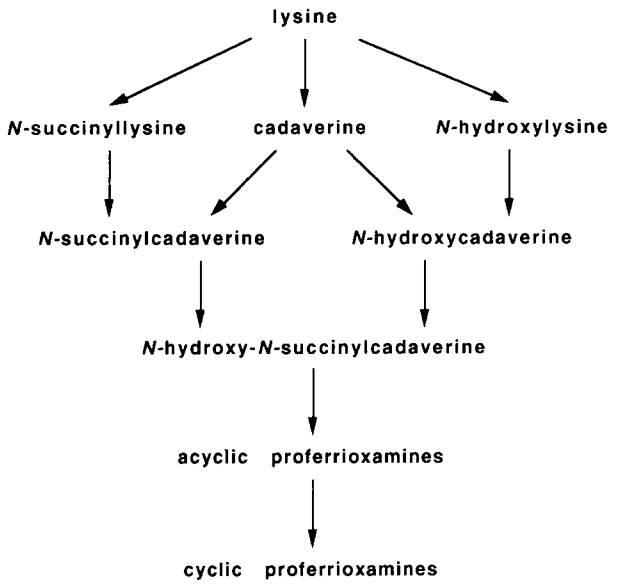

Figure 5. Alternative pathways for the biosynthesis of proferrioxamines from basic amino acids. Theoretically, the first step in the conversion of basic amino acids to proferrioxamines may either be decarboxylation, succinylation or *N*-hydroxylation. While shown here for lysine only, the same scheme also applies to the precursors diaminobutyric acid, ornithine and, in modified form, arginine. A single or all of these pathways may be active in *E. amylovora*. Thus far, evidence has only been gathered for the primary decarboxylation pathway (Feistner 1994d and this study). The other biosynthetic pathways remain hypothetical.

Effect of Hyl on proferrioxamine biosynthesis

Prior to the [$^{15}N_2$]lysine experiment it was attempted to establish lysine as a bona fide precursor of proferrioxamines by feeding Hyl. Other than expected, Hyl proved to be growth inhibitory for *E. amylovora*. This is described in more detail elsewhere (Feistner 1994a,d). The growth inhibition could be reversed by lysine, arginine, ornithine and agmatine, whereas it was not reversible by cadaverine or putrescine. To test whether this inhibition was in any way related to proferrioxamine biosynthesis and iron

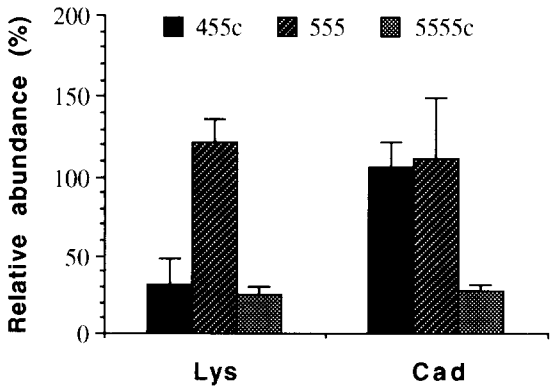

Figure 6. Quantitative comparison of the incorporation of Lys and Cad. Shown are the relative abundances of pFO_{455c}, pFO_{555} and pFP_{5555c}, normalized to the internal standard, pFO_{555Ac} (relative abundance of $pFO_{555Ac} = 100\%$). Within experimental error, there were no differences in the levels of group 1 proferrioxamines between the Lys and the Cad study; however, the abundance of pFO_{455c} was significantly lower in the Lys-supplemented culture.

metabolism in *E. amylovora*, two Hyl-inhibited cultures were supplemented with ferric citrate. Initially a final ferric ion concentration of 2×10^{-6} M was tested, but when no growth was observed for 2 weeks, the ferric ion concentration was increased to 10^{-4} M. Thereafter, one culture grew within 11, the other within 22 days. Concurrently started Hyl/Cad- and Hyl/Put-supplemented cultures did not grow within the observation period of 26 days. This suggested that the mode of action by Hyl may indeed be related to iron metabolism in *E. amylovora*. Because of the long incubation times, contamination with air-borne bacteria was initially a concern; however, this concern was dismissed after a control streak on LB plates produced uniform *E. amylovora*-like colonies and metabolic profiling confirmed the presence of 6-TG (see Materials and methods).

The effect of Hyl, either alone or in combination with Lys, Arg, Orn or Agm, on the proferrioxamine profiles of *E. amylovora* was thus investigated. Data relating to the antagonism of Hyl and Lys are summarized in Table 1. Consistent with the expectation that the slower growth in the Hyl-containing cultures might be due to interference with proferrioxamine biosynthesis, only minor quantities of proferrioxamines were detected in the cultures that were supplemented with 2 p.p.m. Hyl. All proferrioxamine concentrations were low but especially so the concentration of pFO_{555c}. This is consistent with the notion that Hyl mainly acts on Lys-related pathways. It was therefore expected that Lys would derepress the Hyl-repressed proferrioxamine biosynthesis, and that was indeed observed.

Table 2 shows a comparison of the proferrioxamine levels in control and Hyl/Arg- or Hyl/Orn-supplemented cultures. As expected, all Hyl/Arg and Hyl/Orn cultures showed significantly lower than normal levels of proferrioxamines, although no linear correlation with the respective growth

Table 1. Quantitative comparison of relevant proferrioxamines in the Hyl Lys study series

Substrate	pFO_{355c}	pFO_{455c}	pFO_{555c}	pFO_{5555c}
MMB Lys 1*	71.0	444.0	2937.0	270.0
MMB Lys 2*	ND	49.0	1586.0	87.0
MMH Hyl 1	ND	13.3	4.2	ND
MMH Hyl 2	ND	14.6	8.7	ND
MMB Hyl Lys	ND	51.0	298.8	32.6

Listed are peak heights (absolute EM signals as multiples of 10^4), obtained on a single day under identical MS conditions. Optical absorbance profiles at 200 nm (not shown), which were done in series with the mass measurements, are consistent with the mass spectral data presented here. The numbering, i.e. Lys 1, Lys 2, etc., refers to duplicate experiments. The concentration of Hyl in the Hyl-only studies was 2 ppm. The Hyl Lys culture contained 2 g l^{-1} of each amino acid. An asterisk indicates that the extract was diluted 1:10 and the actual measured peak heights were 1 10th of the values given in the Table. ND, not detected.

delays was seen. A shift towards group 2 proferrioxamines was again observed, but in this case the shift was not solely due to the action of Hyl but was also a consequence of the feeding of putrescine precursors. It is interesting, however, that pFO_{444c}, which does not require Lys for its synthesis and which was detected in considerable amounts in the Arg, Agm- and Orn-feeding studies, was absent in the Hyl/Arg and Hyl/Orn cultures. *E. amylovora* obviously was unable to compensate for the loss of cadaverine-containing proferrioxamines by overproducing solely putrescine-based proferrioxamines. Perhaps this can be explained by attributing two modes of action to Hyl; one being the blocking of lysine incorporation into proferrioxamines, the second being the downregulation of putrescine-based pathways such as was observed in the lysine feeding study (see above).

Conclusions

It was unambiguously shown that $[^{15}N_2]$lysine and $[^{13}C_6]$arginine are bona fide precursors for proferrioxamines in *E. amylovora*. The precursor function of ornithine and diaminobutyric acid was not proven in the same stringent manner but is highly likely on grounds of analogous biosynthetic pathways and the observation of predictable shifts in the proferrioxamine profiles following supplementation of the bacterial cultures with these amino acids. The successful incorporation of diaminohexane and hydroxyputrescine furthermore proves that diamines can be biosynthetic precursors for proferrioxamines. Taken together, this estalishes one biosynthetic pathway for proferrioxamines in *E. amylovora*, i.e. the sequence basic amino acids–diamines–proferrioxamines. The incorporation of basic amino acids into proferrioxamines via pathways other than initial decarboxylation cannot be excluded. In fact, the inability to prove preferential incorporations of cadaverine over lysine, and the apparent ability of lysine to reverse the Hyl-induced growth inhibition, while cadaverine

Table 2. Quantitative comparison of relevant proferrioxamines in the Hyl/Orn/Arg study series

Substrate	growth on day	pFO_{445c}	pFO_{355c}	pFO_{455c}	pFO_{555c}
MMB 1	3	12.8	15.7	166.4	168.5
MMB 2	3	44.9	44.7	320.6	351.7
MMH/Hyl/Arg 1	4	ND	ND	6.8	ND
MMB/Hyl/Arg 2	5	8.5	6.4	83.7	37.9
MMB/Hyl/Orn 1	6	7.9	ND	21.6	ND
MMB/Hyl/Orn 2	6	3.0	ND	12.5	ND

Presented are peak heights (absolute EM signals as multiples of 10^4), obtained on a single day under identical MS conditions. The numbering, i.e. MMB 1, MMB 2, etc., refers to duplicate experiments. Optical absorbance profiles at 200 nm (not shown), which were done in series with the mass measurements, are consistent with the mass spectral data presented here. A reduction of proferrioxamine biosynthesis in the presence of Hyl, which can be only partly reversed by arginine or ornithine, is evident. The proferrioxamine levels were also low in the Hyl/Agm culture supernatants (growth observed on days 7 and 11, respectively) but data for the latter are not included in this Table, because they were obtained on a different day. No pFO_{444c} was seen in any of the supernatants.

is unable to do so, provide justification to search for the existence of such alternative pathways.

The finding that Hyl is both, an effective growth inhibitor for *E. amylovora* (Feistner 1994d) and an inhibitor of proferrioxamine biosynthesis, is of considerable interest because it suggests that control of fire blight might be possible with inhibitors of proferrioxamine biosynthesis. The molecular mechanisms behind the Hyl-induced inhibition of growth and proferrioxamine synthesis still must be determined. At this point it is not clear whether the reduction in proferrioxamine levels is the cause or the result of the slow growth of *E. amylovora* in Hyl-containing media. If it can be unambiguously proved that the growth inhibition is the result of interference with proferrioxamine biosynthesis, this will trigger efforts to rationally design fire blight control agents based on this principle, and also provide a strong rational to search for competitive siderophores among the many strains of *E. herbicola* and *Pantoea* spp. that are inhibitory for *E. amylovora* (Johnson *et al.* 1993, Wilson & Lindow 1993, Wodzinski *et al.* 1994). The fermentative production of such competitive siderophores, if they exist, may be commercially viable and may eliminate the poor reproducibility that is inherent in the biocontrol of fire blight when using living organisms. It is expected that mass spectrometry will continue to play a prominent role in all these various endeavors.

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