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Abstract

Pseudomonas aeruginosa is a human opportunistic pathogen ubiquitously distributed in nature. Under iron limiting conditions, this organism secretes pyoverdine which helps to chelate iron to form ferripyoverdine complexes recognized at the outer membrane by ferripyoverdine receptors, these receptors help to transport iron bound to pyoverdine into the internal milieu. This work used P. aeruginosa wildtype and ferripyoverdine receptor mutants (for the first time) cultures as inoculums for the VITEK 2 (bioMerieux) biochemical identification system to study the possible role ferripyoverdine receptors might play in the ability of P. aeruginosa to utilize substrates impregnated in the VITEK 2 GNI cards, in the presence of iron and gentamicin, differential utilization of substrates were observed for P. aeruginosa wildtype and ferripyoverdine receptor mutants.

Introduction

Pseudomonas aeruginosa is pathogenic both for humans and plants. P. aeruginosa causes infections in patients which are immunosuppressed such as burn wound or cancer patients and it is often found to colonize the lungs of cystic fibrosis patients (Terada et al. 1999, Oliver et al. 2000). P. aeruginosa under iron limiting conditions produces compounds called siderophores (Neilands,1995) and the two major siderophores produced are pyoverdine and pyochelin, both having different affinities for iron, iron chelated by these siderophores are recognized at the outer membrane by ferrisiderophore transporters. In addition to these two siderophores, P. aeruginosa has been observed to be growth stimulated by siderophores produced by other bacteria and fungi and several transporters have been identified in its genome which are expressed for these heterologous siderophores (Meyer, 1992; Cornelis and Matthijs, 2002).Three pyoverdine types are secreted by P. aeruginosa (pyoverdine types I-III) and the ferripyoverdine transporters FpvA and FpvB (alternative ferripyoverdine transporter) recognizing each bound to iron has been characterized (Poole et al. 1993; De Chial et al., 2003; Ghysels et al. 2004). Pyoverdine production and type produced (siderotyping) in addition to nutrient utilization assays have been used to group these organisms (Meyer et al. 1997; Vandamme et al. 1996). VITEK 2 (bioMerieux, Inc. Hazelwood, MO) is an automated antibiotic susceptibility testing and microbiology identification system that evaluates an optical signal generated by individual biochemical reactions contained within a variety of microbe identification cards, bacterial suspensions made are used for inoculation of these cards which are incubated for some hours and read by the instrument's internal optics (Hata et al. 2007). The accuracy of this system has been tested (Barry et al. 2003; Sanders et al. 2000) and an enhancement of its performance has also been proposed following several evaluation studies (Saegeman et al. 2005). Reports in the past involving the ability of P. aeruginosa to hydrolyze amino acids has been documented (Riley and Behal, 1971). The importance of certain amino acid residues in some toxic metabolites (exotoxin A) of P. aeruginosa and the probable use of this knowledge as an avenue of drug discovery was proposed (Wozniak et al. 1988), because of the similarities which maybe involved in amino acid transport and ferripyoverdine transport, a possible involvement of ferripyoverdine receptors in metabolism of P. aeruginosa is proposed in this work which used P. aeruginosa wildtype (PAO1) and its ferripyoverdine receptor mutants (PAO1-pvdD pchEF FpvA , PAO1-pvdD pchEF FpvB, and PAO1-pvdD pchEF FpvAFpvB) cultures in the presence of iron and gentamicin as inoculums for the VITEK 2 Biochemical identification system and the purpose of this work was to study the possible role these receptors might play in regulating metabolism in P. aeruginosa.

Materials And Methods

Strains used in this study. PAO1 is a wildtype, type I pyoverdine producing strain which produces pyoverdine under conditions of iron limitation, PAO1-*pvdD pchEF FpvA*, PAO1-*pvdD pchEF FpvB*, and PAO1-*pvdD pchEF FpvAFpvB* are type I pyoverdine negative mutants constructed as previously described (Lamont *et al.* 2002; Ghysels *et al.* 2004). These mutants were constructed following a siderophore-free background created in *P. aeruginosa* PAO1 by making

unmarked deletions in the *pvdD* (pyoverdine biosynthesislocus) and *pchEF* (pyochelin biosynthesis operon). The mutation in *fpvA* delayed, but did not abolish growth in the presence of type I pyoverdine, following the observation that an *fpvA* mutant could still grow in the presence of type I pyoverdine, an alternative receptor called FpvB was identified, correspondingly, inactivation of this alternative receptor led to observable growth in the presence of type I pyoverdine, however, no growth is detected following an inactivation of both receptor genes.

See Illustration 1

VITEK Gram negative identification (GNI) card. The VITEK gram negative identification systems involved the use of cards impregnated with a variety of substrates and the VITEK 2 system GNI card (bioMerieux Inc., Hazelwood, MO, Version 02.01n) used in this study comprised of the following (abbreviated) biochemical details: APPA, H_2S, BGLU, ProA, SAC, ILATk, GlyA, 0129R, ADO, BNAG, dMAL, LIP, dTAG, AGLU, ODC, GGAA, PyrA, AGLTp, dMAN, PLE, dTRE, SUCT, LDC, IMLTa, IARL, dGLU, dMNE, TyrA, CIT, NAGA, IHISa, ELLM, dCEL, GGT, BXYL, URE, MNT, AGAL, CMT, ILATa, BGAL, OFF, BAlap, dSOR, 5KG, PHOS, BGUR (see abbreviations below). Prepared bacterial suspension was used as inoculum and following inoculum's dilution and adjustment of the optical density reading to a standard value, the GNI card was then loaded in the incubator and optical reading, data transmission and card disposal processes were carried out by the instrument. In this study, *P. aeruginosa* wildtype and ferripyoverdine receptor mutants PAO1, PAO1-*pvdD pchEF FpvA*, PAO1-*pvdD pchEF FpvB*, and PAO1-*pvdD pchEF FpvAFpvB* (as previously described under the subheading: strains used in this study and also by Ghysels *et al.* 2004) were grown on LB agar overnight at 37°C. Freshly grown cultures were used as inoculums prepared as suspensions from cotton swab of sample bacteria (*P. aeruginosa* wildtype and ferripyoverdine receptor mutants) applied directly to 3ml of sterile water or sterile water of same volume containing either 100µM iron or 100µg/ml gentamicin or both, adjusted to a turbidity of about 0.55 and the VITEK 2 instrument automatically filled, sealed, and incubated the individual test cards with the prepared culture suspension. The concentration of gentamicin (100µg/ml, Ghysels *et al.* 2004) was also used in the reaction mixture for wildtype (PAO1) and ferripyoverdine receptor double mutant (PAO1-*pvdD pchEF FpvAFpvB*). Optical readings are taken automatically and based on these readings, an identification profile was established and interpreted according to a specific algorithm. Results obtained were compared to the database, generating an identification listed as "excellent," "very good," "good," "acceptable," or "low discrimination" (which are considered correct) for an unknown organism.

Results

P. aeruginosa wildtype and ferripyoverdine receptor mutants were identified as shown (Table 1) with 97-98% confidence intervals and in the presence of gentamicin and iron were observed to test positive or negative for 47 substrates impregnated in the VITEK biochemical identification card (Biochemical substrate Table 2). If these results are actually what they are confirmed to be, an enhancement of the VITEK system may just be ideal to understand various biochemical processes in different microorganisms and this identification system would have to be validated with other experimental conditions and pathogenic organisms.

See Illustration 2

Abbreviations

APPA (Ala-Phe-Pro-ARYLAMIDASE),
LIP (LIPASE),
dMAN (D-MANNITOL),
TyrA (Tyrosine-ARYLAMIDASE),
URE (UREASE),
ILATa (Assimilation L-LACTATE),
BGAL (BETA-GALACTOSIDASE),
O129R (RESISTANCE O/129 comp. vibrio),
dTRE (D-TREHALOSE),
CMT (COUMARATE),
AGLTp (Glutamyl Arylamidase pNA),
IHISa (Assimilation L-HISTIDINE),
BALap (BETA-Alanine arylamidase pNA)
WT (Wildtype), Fe (Iron).

Discussion and Conclusion

Iron plays important role in Pseudomonas aeruginosa, a number of genes are iron regulated in this organism (Ochsner et al. 2002). Nutrient utilization studies and enzyme assays have been used in the past to characterize mutants (Berg and Shaw, 1981), nutrient profiling assays have also been used to characterize microorganisms (Vandamme et al.1996). P. aeruginosa is known to secrete a number of virulence factors which enables colonization and effective

infection of the human host, examples of these virulence factors are cell-bound virulence factors like pilin, flagellae, lipopolysaccharide, adhesins and alginate, secreted virulence factors (exotoxins and proteases), other enzymes like adenylate kinase and phosphate kinase have also been documented as virulence factors of these organisms (Park et al. 2000; Markaryan, et al. 2001; Terada et al. 1999; Rashid et al. 2000). Pyoverdine has also been shown to be a virulence factor (Meyer et al. 1996).

Studies in the past demonstrating the uptake of amino acids by lipid of P. aeruginosa for instance, showed that resting cells of this organism varied in their ability to utilize different amino acids, as opposed to what was observed for metabolically active cells (Silberman and Gaby, 1961). P. aeruginosa has been documented to be highly adaptable organisms, able to grow on a variety of substrates and alter their properties in response to changes in the environment (Lambert, 2002); does gentamicin and or iron contribute to the metabolic activity of these organisms as regards the differential substrate utilization observed for wildtype and ferripyoverdine receptor mutants of P. aeruginosa?

APPA (Ala-Phe-Pro-Arylamidase) is one of those enzymes whose effect was found to be regulated by iron (Table 2). Arylamidases are intracellular enzymes used by P. aeruginosa for amino acid hydrolysis (Riley and Behal, 1971). Riley and Behal studied the influence of media composition (TYE and GBS) on bacterial production of arylamidase, the tryptose-yeast extract (TYE) medium contained 10 g of tryptose, 1.5 g of yeast extract, and 0.5 g of sodium chloride per litre of distilled water while the glucose-basal salts (GBS) medium contained I g of dipotassium phosphate, 0.5 g of sodium chloride, 0.5 g of magnesium chloride, 0.1 g of manganous chloride, 0.1 g of ferrous chloride, 2 g of ammonium sulphate, and 5 g of glucose per litre of distilled water , in addition, differential utilization of substrate was observed in these two media, could the differential utilization of substrate observed for P. aeruginosa cells in the different media be due to the presence of iron in one media and not in the other? Was the arylamidase activity observed dependent on iron? Ferripyoverdine receptors are iron regulated outer membrane proteins (IROMP) expressed when P. aeruginosa cells are grown in iron limiting medium, can arylamidases be compared to ferripyoverdine receptors? This comparison is based on the outcome of the result from the VITEK experiment (Table 2), whereby only the ferripyoverdine receptor mutant (PAO1-pvdD pchEF FpvB) in the presence of iron tested positive for this enzyme activity. Can it be said that arylamidases are iron regulated genes or the effect of iron on this gene is indirect via the ferripyoverdine receptor? In addition, from the experiment of Riley and Behal, there was also a further indication that some particular protein(s) must be synthesised for P. aeruginosa cells grown in the GBS medium to become capable of transporting amino acid, can one of these proteins be ferripyoverdine receptor? Can the process of amino acid transport and iron uptake be linked together in P. aeruginosa? Ferripyoverdine transport process is an energy dependent process, there was also an observation that the process of amino acid uptake into P. aeruginosa was also energy dependent, are both energy systems coupled or one is dependent upon the other? Riley and Behal hypothesized that the possibility of the low levels of recoverable aylamidases activity in the early phases of growth of P. aeruginosa in the different media (TYE and GBS media) used may be due to the result of the enzyme being bound to a membranous material and thereby prevented from functioning, ferripyoverdine receptors are found at the outer membrane level of P. aeruginosa, is ferripyoverdine receptor the membranous material?

P. aeruginosa is known to be in possession of a number of antibiotic resistance systems, one of which is gentamicin (Smith et al. 1975; Korfhagen et al. 1976), the activities of the following enzymes or substrates were influenced by gentamicin alone or in combination with iron, (Table 2) , IHISa (assimilation histidine), ILATa (assimilation L-Lactate), dTRE (D-Trehalose).

Exotoxin A, is a virulence factor and a toxic metabolite of P. aeruginosa which inactivates elongation factor 2 (EF-2) involved in eukaryotic protein synthesis, the importance of His (His-426) has been reported (Wozniak et al. 1988), and Wozniak et al. 1988 postulated that modification of any or all of these residues (His-Glu-Trp) will result in the production of an immunologically cross reactive but inactive toxin, and this at that time was proposed to be a target for vaccine development. Cationic antibiotics (gentamicin) increase the permeability of the outer membrane by disrupting the outer membrane structure allowing free penetration of compounds (Peterson et al. 1985), gentamicin alone or in combination with iron could influence the use of histidine by P. aeruginosa wildtype and ferripyoverdine receptor mutants PAO1, PAO1-pvdD pchEF FpvA, PAO1-pvdD pchEF FpvB, and PAO1-pvdD pchEF FpvAFpvB, can this knowledge be useful with respect to ferripyoverdine receptors in the area of drug discovery against P.

aeruginosa and other human opportunistic pathogens?

Enzymes are capable of chemical modifications and they have been studied to modify the physicochemical properties of a reaction medium, (Chopineau et al. 1989), can the differential enzyme activities observed be due to the effect of iron or gentamicin or a modified reaction environment? These questions may be answered in further research; the data provided in this study may serve as preliminary for other future research. Mutation or no mutation, the strain or plasmids used in this study were identified as “Pseudomonas aeruginosa” with 97-98% confidence intervals. This goes to show that the VITEK 2 system for microbiology identification can also be applied not only to wildtype strains, but also to mutants. For all the enzymes or substrates whose activities were influenced by gentamicin or iron or both , complementation experiments will be carried out and it maybe interesting to link the observation from these experiment to ferripyoverdine receptors, this work, which will be done in the near future will help improve the already vast knowledge on the ferripyoverdine receptors of P. aeruginosa and this may then later show that the receptors may not only be serving as entrance route for antibiotics, ferri-siderophores and other lethal compounds (Osayande, J,O. 2013) but also as regulators of general metabolism in this organism.

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Illustrations

Illustration 1

Table 1: VITEK gram negative identification of P. aeruginosa wildtype and ferripyoverdine receptor mutants strains used in this study.

Strain	Reference	Identification	Confidence
PAO1	Stover *et al.* 2000	98% probability *Pseudomonas aeruginosa*	Excellent identification
PAO1-*pvdD pchEF FpvA*	Ghysels *et al.* 2004	97% probability *Pseudomonas aeruginosa*	Excellent identification
PAO1-*pvdD pchEF FpvB*	Ghysels *et al.* 2004	97% probability *Pseudomonas aeruginosa*	Excellent identification
PAO1-*pvdD pchEF FpvAFpvB*	Ghysels *et al.* 2004	97% probability *Pseudomonas aeruginosa*	Excellent identification

Illustration 2

Table 2: Biochemical substrate utilization table

Strains	APPA	LIP	dMAN	TyrA	URE	ILATa	BGAL	O129R	dTRE	CMT	AGLTp	IHISa	BALap
PAO1 (WT)	-	+	+	-	+	-	-	+	-	+	-	-	+
PAO1 (WT) + Gentamicin	-	+	-	+	-	-	-	-	-	+	-	-	-
PAO1 (WT) + Fe	-	-	-	+	-	+	-	+	-	+	+	+	+
PAO1 (WT) + Fe + Gentamicin	-	-	-	+	-	-	-	+	-	+	-	-	-
PAO1-*pvdD pchEF FpvA*	-	-	+	+	+	-	-	+	-	+	+	-	+
PAO1-*pvdD pchEF FpvA* + Gentamicin	-	-	+	+	-	+	-	+	-	+	-	+	+
PAO1-*pvdD pchEF FpvA* + Fe	-	-	-	+	+	-	-	-	-	-	+	-	+
PAO1-*pvdD pchEF FpvA* + Fe + Gentamicin	-	-	-	+	+	-	-	-	-	-	-	-	+
PAO1-*pvdD pchEF FpvB*	-	+	+	-	+	-	-	+	-	+	+	-	+
PAO1-*pvdD pchEF FpvB* + Gentamicin	-	+	+	-	-	+	-	+	-	+	-	-	+
PAO1-*pvdD pchEF FpvB* + Fe	+	-	-	+	-	+	+	+	+	-	+	-	+
PAO1-*pvdD pchEF FpvB* + Fe + Gentamicin	-	-	-	+	-	+	-	-	+	-	+	-	+
PAO1-*pvdD pchEF FpvAFpvB*	-	-	+	+	+	-	-	+	-	+	+	-	+
PAO1-*pvdD pchEF FpvAFpvB* + Gentamicin	-	-	-	+	+	-	-	-	-	+	-	-	+
PAO1-*pvdD pchEF FpvAFpvB* + Fe	-	-	-	+	+	-	-	-	-	-	+	-	+
PAO1-*pvdD pchEF FpvAFpvB* + Fe+ Gentamicin	-	-	-	+	-	+	-	+	-	+	-	+	+

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