

## Research Paper

## Auxotrophy in natural isolate: minimal requirements for growth of the Antarctic psychrotrophic bacterium *Pseudomonas syringae* Lz4W

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A chemically defined minimal medium has been developed for growing the Antarctic psychrotrophic bacterium *Pseudomonas syringae* Lz4W, a model system for studying cold adaptation. This natural isolate from Antarctica has an absolute requirement for two branched chain amino acids, isoleucine and valine, in addition to low osmolality of the growth medium. The bacterium contains threonine deaminase but lacks acetoxyacid synthase suggesting that a defect lies in the isoleucine and valine biosynthetic pathway causing auxotrophy. Succinate was found to be preferred carbon source over glucose as it could suppress the glucose metabolizing enzymes in the cells, like in other pseudomonads. The development of the minimal medium (MM<sub>Lz</sub>) for growing the Antarctic *P. syringae* Lz4W strain would be useful for investigation of the catabolite repression control mechanism at a very low temperature (below 5 °C), which is predominant in vast area of our global ecosystems.

**Keywords:** Antarctic bacterium / *Pseudomonas syringae* Lz4W / Minimal growth medium / Amino acid auxotrophy

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### Introduction

The psychrotrophic bacterium *Pseudomonas syringae* Lz4W that was isolated from the Schirmacher oasis Antarctica [1] has been used as a model system to understand the cold adapted physiology of microorganism [2–14]. Interest in the physiology and molecular biology of psychrotrophic and psychrophilic bacteria, which have the ability to grow at a low temperature (0–4 °C), is gaining importance because of the lack of knowledge about their physiology, and owing to their importance in global ecology, as vast area (~80%) of our globe experiences the temperature below 5 °C [15, 16]. Additionally, low temperature adapted microorganisms have immense biotechnological potentials [17, 18]. Discovery of psychrophilic enzymes of industrial importance, cleaning organic pollutants from the cold environments, combating refrigerated food-spoiling

bacteria and cold-adapted pathogenic bacteria have provided exciting challenge for their studies in recent years. We are just beginning to get insight into the molecular basis of novel strategies that the psychrotrophic and psychrophilic microorganisms have evolved for growth at low temperature [5, 16, 17, 19, 20].

Pseudomonad group of bacteria is known for its catabolic versatility of wide range of organic carbon source [21–24]. Extensive research on the catabolism of different organic and aromatic compounds by different mesophilic *Pseudomonas* species including *P. putida*, and *P. aeruginosa*, and *P. fluorescens* have been reported [23, 24]. However, information on the catabolic control of organic and aromatics carbon utilization in cold environment is scarce [25]. In our quest for understanding the catabolite control mechanism at low temperature environment we wish to use the Antarctic *P. syringae* as the organism of our choice. The bacterium grows under laboratory conditions in complex medium containing peptone and yeast extracts at the temperature range of 0 °C to 30 °C with optimum growth at ~22 °C. However, a minimal medium with defined chemical composition is indispensable for the study of catabolic physiology of

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carbon and nitrogen sources, and identifying factors that are responsible for the regulation. In this respect, several minimal growth media have been used for different *Pseudomonas* species, which include M9 salts medium, MOPS minimal medium, and basal mineral medium [26–28]. To our surprise, *P. syringae* Lz4W could not be grown in these growth media. We noticed that very little information is available on the growth of psychrotrophs and psychrophiles in a defined minimal media. Therefore, we undertook this study to determine the minimal nutritional requirement of the *P. syringae* Lz4W for growth. We report here the auxotrophic nature of the Antarctic bacterium, and the composition of a defined minimal medium (MM<sub>Lz</sub>) that would support the growth of *P. syringae* Lz4W at low (4 °C) and high (22 °C) temperatures. We also discuss the significance of auxotrophy in natural environment, which has ecological implication as well as in evolutionary biology.

## Materials and methods

### Bacterial strain and growth media

Isolation of *P. syringae* Lz4W from the Schirmacher oasis of Antarctica has been reported earlier [1]. Stock cultures were kept frozen in 15% glycerol at –70 °C, as well as under lyophilized condition for longer storage. The bacterium is routinely grown at 4 °C and 22 °C in Antarctic bacterial medium (ABM) in refrigerated incubator shaker (New Brunswick Scientific, Edison, NJ), or on ABM-agar plates. ABM contains 0.2% yeast extract and 0.5% peptone, about half of the nutrient strength of Luria-Bertani medium but without sodium chloride. Media pH was adjusted to desired value using either NaOH or HCl. Composition of standard Minimal M9 medium and basal salt medium was as described [29]. We omitted NaCl and CaCl<sub>2</sub> from M9, which we would refer to as M8, as a starting point of determining the growth requirements. The finally determined minimal medium (MM<sub>Lz</sub>) for *P. syringae* had the following composition: 8.45 mM Na<sub>2</sub>HPO<sub>4</sub>, 4.41 mM KH<sub>2</sub>PO<sub>4</sub>, 3.79 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.0 mM MgSO<sub>4</sub>, valine and isoleucine (0.01%, each), and 1% desired carbon source (usually succinate).

All chemicals including amino acid, vitamins, carbon sources, and inorganic salts were of analytical grade unless stated otherwise, and obtained mainly from Sigma-Aldrich and Merck. Yeast extract, peptone and agar were bought from Hi-Media (India). Stock solution of carbon sources and amino acids were filter-sterilized (0.22 µm pore size, Millipore filter) before use.

### Growth condition and assessment of growth

Batch cultures were grown in Erlenmeyer flasks in a refrigerated incubator shaker with agitation (250 rpm) at the desired temperatures. Generally, starter culture was set up from single colony isolates into 3 ml of ABM and grown overnight at 22 °C, which was then inoculated at a 1% dilution into 25 ml of the broth in 150 ml flask. Growth was monitored by the turbidity (optical density) of cultures measured at 600 nm in a spectrometer (UV-1601 model, Shimadzu, Japan). Data for growth yield in different media reported in this study are generally the means of triplicate OD<sub>600</sub> values of the culture repeated twice. Statistical analysis and growth curves were plotted using Graphad Prism 4.0 software.

### Osmolality of growth medium

Reported osmolality of growth medium in this study was not experimentally determined, but calculated based on the equation: osmolality =  $\Phi \times n \times C$ , where  $\Phi$  is the osmotic coefficient and accounts for the degree of dissociation of the solute,  $n$  is the number of particles (ionic species) into which a molecule dissociates, and  $C$  is the molar concentration of the solution [30]. Experimentally, osmolality of minimal media was changed by altering only the concentration of Na<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, and MgSO<sub>4</sub>, but keeping the constant amount of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as nitrogen source (0.05% or 3.79 mM), 1% carbon source (e.g., succinate), and obligatory requirements of isoleucine and valine (0.01%, each). Thus, for M8 medium, Na<sub>2</sub>HPO<sub>4</sub> (42 mM) generating 3 ionic species (two Na<sup>+</sup> and one HPO<sub>4</sub><sup>-</sup>) would contribute 3 × 42 milliosmole or 126 mOsm, KH<sub>2</sub>PO<sub>4</sub> (22 mM) with two ionic species (K<sup>+</sup> and H<sub>2</sub>PO<sub>4</sub><sup>-</sup>) would contribute 2 × 22 = 44 mOsm, and 1 mM MgSO<sub>4</sub> with two ionic species contributing (2 × 1) or 2 mOsm to produce a total of 172 mOsm/kg of water. The presumption here is that the value of  $\Phi$  is 1, i.e., 100% dissociation of the osmolytes in the growth medium. Using this calculation M8 (1X) medium constituting 172 mOsm was increased to higher strength, such as 2X giving 344 mOsm, and 3X giving 516 mOsm, or decreased to produce 1/5<sup>th</sup> (34.4 mOsm), 1/10<sup>th</sup> (17.2 mOsm), and 1/50<sup>th</sup> (3.44 mOsm) strength. Since 3.79 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was kept constant in the *P. syringae* Lz4W minimal medium (MM<sub>Lz</sub>), this salt would add up additional (3 × 3.79) or 11.4 mOsm in each case. Carbon source, or isoleucine (I) and valine (V) were not considered for osmolality calculation.

### Utilization and optimization of different nutrients

All carbon source utilization was determined at a concentration of 1% (w/v) added to the MM<sub>Lz</sub> constituents

and at a pH 7.0. Optimal requirement of succinate was determined by examining growth rate by varying its concentration (0, 0.2, 0.5, 1, and 5 %) in MM<sub>Lz</sub>. Optimal requirement of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as nitrogen source was determined in M8 medium lacking the ammonium salt but containing 0.01% of each of I and V, 1% succinate, and varying concentration of the salt. Mostly, in these experiments the bacterium was grown at 22 °C, unless otherwise specified.

### Determination of enzyme activities

Enzyme assays for glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) and Entner-Duodoroff pathway enzymes, Edd (EC 4.2.1.12) and Eda (EC 4.1.2.14) were carried out with the cell extracts of *P. syringae*. Extracts were prepared from late exponential phase cells cultured in MM<sub>Lz</sub> containing glucose or glucose plus succinate. Cells were disrupted by ultrasonication as reported [8] and then cell debris was removed by centrifugation at 15000 rpm. The supernatant was used for the NADP- and NAD-linked spectroscopic assays of the enzyme activities [26, 31]. Briefly, for G6PDH assay, 100 µl reaction volume contained 50 mM Tris-HCl pH 8.2, 10 mM MgCl<sub>2</sub>, 1 mM NADP, and 10 mM glucose-6-phosphate (G6P). Cell extract was added to start reaction and the rate of NADP reduction was monitored at OD<sub>340</sub>. For the combined activity of Edd (6-phosphogluconate (6-PG) dehydratase) and Eda (2-keto-3-deoxy-6-phosphogluconate aldolase), 100 µl reaction volume contained 50 mM Tris-HCl pH 8.2, 10 mM MgCl<sub>2</sub>, 0.1 mM NADH, 20 mM 6-PG, and excess amount of lactate dehydrogenase (300 µg). The oxidation of NADH was monitored by the increase of OD<sub>340</sub> in the reaction. One unit of enzyme activity has been expressed as nmole of substrates (NADP or NADH) converted per minute per milligram of protein.

The cellular activity of biodegradative threonine deaminase (TD) (EC 4.3.1.19), which converts L-threonine into α-ketobutyrate, was determined colorimetrically [32]. One unit of the enzyme has been expressed as micromole of α-ketobutyrate produced per minute per milligram of protein. Protein was estimated by Bradford method [33].

### Microscopic determination of cell size

Bacterial cell size was determined as reported earlier [14] from phase contrast images using a Carl Zeiss microscope (Axioplan 2 imaging) with inbuilt axiovision ver.3.1 software. A minimum of 200 cells from ~10–15 visual fields were used for the calculation of cell size.

## Results

### *P. syringae* Lz4W is an amino acid auxotroph

As a basis of our minimal media for growth of *P. syringae* Lz4W we used a modified basal M9 medium, called M8 that contains Na<sub>2</sub>HPO<sub>4</sub> (42 mM), KH<sub>2</sub>PO<sub>4</sub> (22 mM), NH<sub>4</sub>Cl (19 mM), and MgSO<sub>4</sub> (1 mM) but lacks CaCl<sub>2</sub> (0.09 mM) and NaCl (9 mM). The latter two salts were omitted as we observed that NaCl inhibits growth, and CaCl<sub>2</sub> inclusion or exclusion does not make any difference to the growth of the bacterium. Next, 0.2% of one of three carbon sources (succinate, pyruvate, and glucose), four vitamins (biotin, 2.05 µM; riboflavin, 1.33 µM; thiamine (2.96 µM) and lipoic acid, 24.0 pM) and 20 amino acids (L-form) were added to the M8 salt medium for studying the growth. This medium allowed the growth of *P. syringae* at both low (4 °C) and high (22 °C) temperatures. Addition or elimination of vitamins did not affect the growth efficiency, and these were therefore removed from the test. It was also observed that succinate among the carbon sources yields better result with the cell density as measured by the OD<sub>600</sub> of the cultures. Therefore, the M8 salt medium containing succinate and 20 amino acids were used as the starting point for elimination of different amino acids (Table 1). Initially, using different sets of amino acids, it was found out that *P. syringae* Lz4W could grow in a 9-amino acids set that contains cysteine (C), methionine (M), tryptophan (W), arginine (R), histidine (H), isoleucine (I), leucine (L), valine (V), and glutamine (Q), similar to the one described for *Listeria monocytogenes* [34]. Using three more subsets (C, M, W, R, H), (W, R, H,

**Table 1.** Amino acid requirements for growth of *P. syringae* Lz4W. Added L-amino acids (0.1%) are indicated by single letter code. SA (succinate) was of 1% (final concentration).

Composition	Growth yield (OD <sub>600</sub> )	
	12 h	24 h
M8 + succinate (SA) + 20 aa	0.57	2.46
M8 + SA + set of 9 aa (C, M, W, R, H, I, V, L, and Q)	0.56	2.21
M8 + SA + set of 7 aa (W, R, H, I, V, L, and Q)	0.44	0.90
M8 + SA + set of 5 aa (C, M, W, R, and H)	0.07	0.08
M8 + SA + set of 4 aa (I, V, L, and Q)	0.69	2.38
M8 + SA + (I + V)	0.51	1.94
M8 + SA + (L + Q)	0.04	0.03
M8 + SA + I	0.05	0.05
M8 + SA + V	0.05	0.05
M8 + SA + L	0.05	0.03
M8 + SA + (20 aa – I)	0.05	0.05
M8 + SA + (20 aa – V)	0.06	0.06

I, V, L, Q), and (I, V, L, Q), we found that the Antarctic *P. syringae* Lz4W could grow proficiently in M8 salt medium containing the last subset of amino acids. By selective deletion of each of the four amino acids from this subset we found that Lz4W requires only the branched-chain amino acids, I and V, in the medium for growth. Both isoleucine and valine together were found absolutely essential. The other branched chain amino acid leucine could not substitute any one of them. Additional experiments by eliminating succinate and increasing the concentration of I and V in the medium suggested that these two amino acids, in addition to their requirement in biosynthesis, could also serve as carbon source in the bacterium.

### pH requirement of growth

Growth of *P. syringae* Lz4W was tested at different pH of the M8 growth medium containing I and V (each 0.01%) and succinate (1%). The bacterium grew between pH 6.0 and 9.0 with optimal growth around pH 7.0. There was no growth at the lower pH 4.0 and 5.0. The bacterium however displayed reduced growth at pH 6.0 and 9.0 when cultured at 4 °C suggesting that low temperature causes additional stress at both ends of the pH. Therefore, the effects of various nutritional requirements in the subsequent study were tested only at pH 7.0.

### Inorganic elements requirement

Since distribution of the elements in soils of different part of Antarctica is not well known, we wanted to examine the essentiality of a few inorganic elements for the growth of *P. syringae* Lz4W. For this, the bacterium was grown in a defined medium that contains the following: M9 phosphate buffer ( $K_2HPO_4$ – $NaH_2PO_4$ ), basal salts ( $FeSO_4$ ,  $NH_4Cl$ ,  $MgSO_4$ ,  $K_2SO_4$ ,  $CaCl_2$ ,  $MgCl_2$ ) and micronutrients (a solution containing ammonium molybdate,  $H_3BO_3$ ,  $CoCl_2$ ,  $CuSO_4$ ,  $MnCl_2$ , and  $ZnSO_4$ ) of concentrations as specified [29], the essential amino acids I and V (each 0.01%), and succinate (carbon source). Each element was then individually deleted by eliminating the chosen salts from the medium and then tested for growth of the bacterium. Removal of  $Na^+$ ,  $K^+$ ,  $Ca^{2+}$ ,  $Co^{2+}$ ,  $Cu^{2+}$ ,  $Mn^{2+}$ ,  $Fe^{2+}$ ,  $Zn^{2+}$ , molybdate and  $BO_3^{3-}$  did not significantly affect the growth. But growth was severely affected when  $Mg^{2+}$ ,  $SO_4^{2-}$ ,  $PO_4^{3-}$  were absent from the media. The  $OD_{600}$  of the cultures in absence of the above three individually reached ~0.3, 0.8, and 0.42 as compare to the control value of 2.3 when all of these inorganic ions were present. Based on these results, we decided to use  $Na_2HPO_4$ ,  $KH_2PO_4$ ,  $(NH_4)_2SO_4$  and  $MgSO_4$  in the minimal medium for the growth of *P. syringae*.

### Nitrogen source optimization

We tested a few common inorganic nitrogen sources including  $NH_4Cl$ ,  $NH_4NO_3$ ,  $(NH_4)_2SO_4$ , and  $KNO_3$  in the minimal media for their effect on growth. Salts (0.2%, w/v) were added to the M8 medium containing 0.001% each of V, and I (ten time less then the usual concentration to minimize the availability of nitrogen from the amino acids) and 1.0% succinate as carbon source. After 24 hours of growth at 22 °C, cultures containing the  $NH_4^+$  salts produced higher cell density ( $OD_{600}$ ) compared to the  $KNO_3$  containing culture. Since  $(NH_4)_2SO_4$  could act as the source for two critical factors, nitrogen and sulfate, it was chosen for determining the optimal requirement. A range of different concentrations (0, 0.05, 0.1, 0.5, and 1.0%) of the salt was tested in the medium that also contained 0.01% each of valine and isoleucine, and 1% succinate. The growth of *P. syringae* Lz4W reduced at higher salt concentration (0.5% and 1.0%), but produced better and almost equal yield with 0.05% and 0.1% of the salt. We have therefore chosen 0.05% (3.79 mM) concentration of  $(NH_4)_2SO_4$  for the final minimal medium.

### Osmolality requirement

The Antarctic *P. syringae* is sensitive to osmolality of the growth media and shows reduced growth when growth medium contains NaCl, such as in LB medium (unpublished observation). We determined the optimal osmolality of the minimal media, starting with the modified M8 that contains 3.79 mM  $(NH_4)_2SO_4$  instead of  $NH_4Cl$  as nitrogen source and had a combined osmolality of ~183.4 mOsm per kilogram of water, as has been described under Materials and Methods. By increasing or decreasing the osmolality we found that the bacterium grows optimally between 1X to 1/5<sup>th</sup> dilution (~45.8 mOsmol) of the M8 salt strength (Table 2). We chose 1/5<sup>th</sup> dilution of the modified M8 for designing the minimal medium (MM<sub>Lz</sub>).

**Table 2.** Effect of osmolality on growth of *P. syringae* Lz4W. The osmolality was changed by altering the concentration of  $Na_2HPO_4$ ,  $KH_2PO_4$ , and  $MgSO_4$  in M8 medium as described under Materials and Methods.

Osmolality (mOsmol)	Growth yield ( $OD_{600}$ )	
	12 h	24 h
527.4	0.18	1.16
355.4	0.29	1.33
183.4	0.4	2.4
45.8	0.49	2.54
28.6	0.37	2.28
14.84	0.32	1.2

### Carbon source utilization

By adding various organic acids, sugars, and alcohols into the above minimal medium we tested the carbon source requirement of the bacterium (Table 3). Among the organic acids, like citrate, succinate, malate,  $\alpha$ -ketoglutarate, fumarate, pyruvate, and acetate all except malate supported the growth. Sodium succinate and fumarate produced better result with comparable growth ( $OD_{600}$  2.24 and 1.91 respectively) while  $\alpha$ -ketoglutarate, pyruvate, citrate, glucuronic acid produced  $OD_{600}$  of 1.18, 0.68, 0.69, 0.82 respectively, after 24 h of growth. Among the sugars (glucose, fructose and sucrose) that were tested only glucose and fructose were utilized, and after 24 h of growth produced  $OD_{600}$  of 1.19 and 0.68, respectively. Among the alcohols, glycerol but not the mannitol was utilized as carbon source. The  $OD_{600}$  of glycerol medium after 24 hour however reached only about 0.76.

Based on the above results sodium succinate was chosen as carbon source in the minimal medium for growing *P. syringae* Lz4W. For determining the optimal amount succinate of different concentrations (0, 0.2, 0.5, 1.0, and 5.0%) were tested. The growth was highest at 1% concentration, yielding  $OD_{600}$  of ~2.55 of the culture after 24 h of growth. Both at lower (0.5%) and higher (5%) succinate concentration bacterial growth were poor;  $OD_{600}$  were 0.67 and 1.31, respectively. Therefore, 1% of succinate was chosen for minimal medium.

Few of the carbon sources (succinate, fumarate,  $\alpha$ -ketoglutarate, citrate, pyruvate, glucose, and fructose) were examined for temperature dependent pref-

**Table 3.** Growth and generation time of *P. syringae* Lz4W in presence of different carbon sources. Carbon sources were added as 1% (final concentration) solution to  $MM_{Lz}$  medium. ND, not determined. None, no C-source added.

Carbon source	Growth ( $OD_{600}$ ) after 24 h at 22 °C	Generation time (h)	
		at 22 °C	at 4 °C
Glucose	1.19 ± 0.19	2.60 ± 0.5	20.0 ± 1.7
Fructose	0.68 ± 0.13	3.91 ± 0.35	24.9 ± 5.7
Sucrose	0.27 ± 0.16	ND	ND
Succinate	2.24 ± 0.31	2.30 ± 0.5	15 ± 1.8
Fumarate	1.92 ± 0.27	2.52 ± 0.08	17.2 ± 1.01
$\alpha$ -ketoglutarate	1.18 ± 0.16	2.84 ± 0.45	12.06 ± 0.09
Malate	0.31 ± 0.18	ND	ND
Pyruvate	0.68 ± 0.28	2.68 ± 0.09	12.9 ± 1.8
Citrate	1.23 ± 0.23	2.1 ± 0.38	14.9 ± 3.02
Acetate	0.75 ± 0.15	ND	ND
Glucuronate	0.82 ± 0.21	ND	ND
Glycerol	0.76 ± 0.2	ND	ND
Mannitol	0.27 ± 0.2	ND	ND
None	0.26 ± 0.15	–	–
ABM	2.6 ± 0.2	1.55 ± 0.26	4.9 ± 0.6

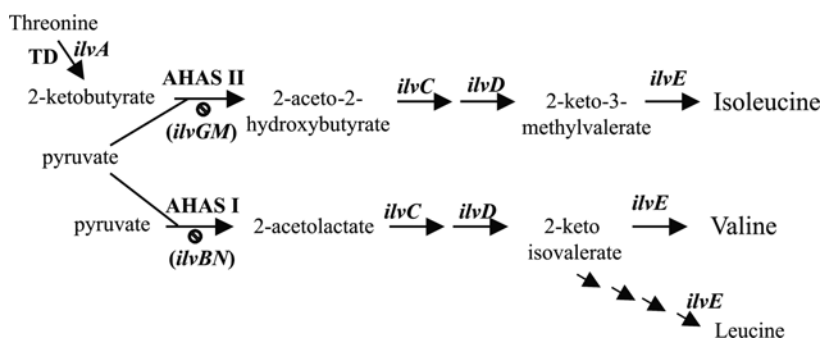
erential utilization, if any, during growth at 22 °C and 4 °C. Doubling time was also determined for both high (22 °C) and low (4 °C) temperatures using these carbon sources (Table 3). In most cases, the growth profiles at 4 °C were similar to that of 22 °C. However, the doubling times in minimal media in the presence of TCA cycle intermediates were shorter when compared with that of glucose and fructose; the effect was more prominent at 4 °C than at 22 °C.

### Amino acid as nutrient source

Twenty L-amino acids were added individually to the medium, which contained minimal salts as specified for  $MM_{Lz}$ , which contained no  $(NH_4)_2SO_4$  and no carbon source but 0.01% each of the essential isoleucine and valine, to examine the amino acids utilization. All the amino acids except glycine, threonine, cysteine, methionine, and tryptophan could provide the C and N requirement for growth of *P. syringae* Lz4W.

### Defect in the *ilv* biosynthesis pathway of *P. syringae*

Inability of *P. syringae* Lz4W to grow in minimal medium without the isoleucine and valine suggests a possible lesion in the biosynthetic *ilv* pathway [35–36] for these two amino acids (Fig. 1). While the main precursor of isoleucine, 2-aceto-2-hydroxybutyrate (AHB), is produced by the condensation of one molecule each of pyruvate and 2-ketobutyrate, the valine precursor 2-acetolactate is produced by the condensation of two pyruvate molecules. Two acetohydroxyacid synthase (AHAS) enzymes are involved in these steps; the production of 2-acetolactate is generally catalyzed by *ilvBN* encoded AHAS isozyme I, and the production of AHB is catalyzed by *ilvGM* encoded AHAS isozyme II. It is important to note that, for isoleucine biosynthesis, a key step before the AHAS II catalyzed step of AHB production includes the conversion of threonine to 2-ketobutyrate by the activity of *ilvA* gene encoded threonine deaminase (TD), the inactivation of which can lead to isoleucine auxotrophy. We measured the TD activity in cells directly to examine the status of this crucial step of the *P. syringae* Lz4W *ilv* pathway. We found that the TD activity is high (~10 unit) when the cells were grown in the  $MM_{Lz}$  medium, suggesting that the *ilvA* gene is functionally active in the bacterium. However, we found that, while threonine and valine together, or 2-keto-butyrate and valine together did not support the growth of the bacterium, the intermediate 2-keto-3-methylvalerate (KMV) of the Ile biosynthesis could allow the growth in presence of valine (Table 4). This suggests that AHAS isozyme II (possibly encoded by the *ilvGM* homologue) catalyzing the ketobutyrate to



**Figure 1.** Isoleucine and valine biosynthetic pathway showing the probable site of lesion (Ø) in the Antarctic *P. syringae* Lz4W.

2-aceto-2-hydroxybutyrate (AHB) is defective or non-functional, but the *ilvE* encoded transaminase is active in the bacterium (Fig. 1). On the other hand, pyruvate and isoleucine also could not support the growth, suggesting that a defect probably also lies in the *ilvBN* (AHAS isozyme I) catalysed step of valine biosynthesis. Subsequent to the initial steps, *ilvC*, *ilvD*, and *ilvE* encoded enzymes work in the parallel pathways for both isoleucine and valine synthesis. Although we could not check the *ilvC* and *ilvD* steps of the pathway due to unavailability of the suitable precursor substrates for growth analysis the data presented in Table 4 suggest that *ilvE* catalyzed step is functional in the bacterium. A detail molecular analysis of the *ilv* genes in *P. syringae* Lz4W would be necessary to identify the exact nature of defects for biosynthesis of the two branched chain amino acids. Interestingly, analysis of genome sequence of plant associated *P. syringae* PV *tomato* and *P. fluorescence* Pfo1 (Acc nos. NC\_004578 and NC\_007492), to which most of the *P. syringae* Lz4W genes show maximum identity (unpublished observation) indicates that both of the former strains contain one each of *ilvD*, *ilvE*, *ilvF*, but two homologues of *ilvA*, one operon for *ilvBNC* but with additional *ilvB* homologues suggesting the redundancy of TD and AHAS enzymes encoded genes in these bacteria and explaining their prototrophic nature.

**Table 4.** Growth of *P. syringae* Lz4W on MM<sub>Lz</sub> containing different combinations of precursors for isoleucine and valine biosynthesis pathway (Fig. 1). Components were added as 0.1% (final concentration) to MM<sub>Lz</sub> containing succinate but without essential isoleucine and valine. '+' and '-' indicates the ability or inability to grow.

Combina- tions	I + V	I	V	T	Pyru- vate	2-keto- butyrate	2-keto-3- methylvalerate
I	+	-	+	-	-	-	-
V	+	+	-	-	-	-	+
T	+	-	-	-	-	-	-
Pyruvate	+	-	-	-	-	-	-

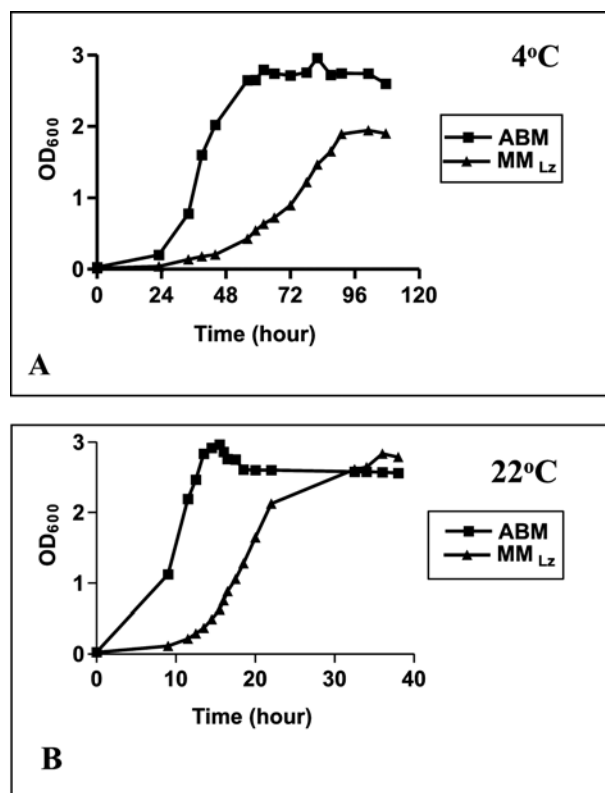
### Growth profile of *P. syringae* at low and high temperature

Analysis of growth curves shows that *P. syringae* Lz4W generally reaches peak cell density approximately around 22–24 hour following growth in MM<sub>Lz</sub> broth at 22 °C. However, peak cell density at 4 °C was seen only after ~105 hour of incubation at the low temperature. Compared to the complex ABM medium growth in MM<sub>Lz</sub> was poor. The doubling time was always shorter (~1.5 h at 22 °C, and 4.8 h at 4 °C) in ABM than in the minimal succinate medium (~2.3 h and 15 h at 22° and 4 °C, respectively). As for the effect of low temperature, growth was about three times slower at 4 °C in the complex ABM and about six times slower when minimal media were used. Most conspicuous feature of the low temperature growth is noticeable in lag period of the growth curves, which is much pronounced in the case of minimal media (Fig. 2). At 22 °C, the lag period is about 9 h for MM<sub>Lz</sub> and 4.5 h for ABM, which enhances considerably to 34 h and 18 h, respectively, at 4 °C.

On plates containing solid agar media, colonies develop only after 52 h of growth in MM<sub>Lz</sub> at 22 °C whereas in ABM colonies are visible after ~27 h of incubation. At 4 °C, the colonies were visible only after 8 and 5 days in MM and ABM, respectively. There was no change in colony morphology on the MM<sub>Lz</sub>- and ABM-agar plates in either of the temperatures.

### Alteration in cell size

Cell size however was found affected in the minimal media. Although both length ( $1.2 \pm 0.1 \mu\text{m}$ ) and breadth ( $0.9 \pm 0.06 \mu\text{m}$ ) of the cells in MM<sub>Lz</sub> reduced as compared to ABM grown cells ( $2.9 \pm 0.3 \mu\text{m} \times 1.1 \pm 0.2 \mu\text{m}$ ) at 22 °C, the effect on length was more pronounced. If one calculates the volume of the cells taking them as cylindrical the change appears to be ~3.6 fold. Interestingly, the *P. syringae* cell size is reduced at low temperature in ABM too [14]. In minimal medium, the smaller cell size is not much further reduced at the low (4 °C) temperature.



**Figure 2.** Growth profiles of *P. syringae* Lz4W in the rich ABM and minimal MM<sub>Lz</sub>-succinate media at low (4 °C) and high (22 °C) temperatures.

### Succinate dependent catabolite repression in minimal media

To examine the suitability of the MM<sub>Lz</sub> medium for carbon catabolite repression study in *P. syringae* we assayed the activity of G6PDH, and the combined activities of Edd and Eda enzymes of the Entner-Duodoroff pathway, which are repressed by succinate in the growth medium in different *Pseudomonas* species [21, 22, 26, 27, 37]. As shown in Table 5, the activities of the enzymes in *P. syringae* Lz4W cell are repressed about 4 fold by succinate in the MM<sub>Lz</sub> + glucose medium. This

**Table 5.** Succinate mediated suppression of glucose metabolizing enzymes in *P. syringae* Lz4W. Specific activity of the enzymes have been shown as nmole of substrate utilized per minute per milligram of protein in cell extract.

Enzyme	Activity in cells grown in		Repression ratio
	MM <sub>Lz</sub> + glucose	MM <sub>Lz</sub> + glucose + succinate	
G6PDH	206.9 ± 3.36	49.3 ± 0.57	~ 4.0
Edd + Eda (combined)	26.24 ± 0.95	6.73 ± 0.45	~ 4.2

is consistent with the succinate as preferred carbon source that represses the expression of glucose metabolizing enzymes.

### Discussion

Information on the catabolic physiology of psychrotrophic/psychrophilic *Pseudomonas* strains is very scarce. Investigation on this aspect using the Antarctic *P. syringae* Lz4W as a model system necessitated the development of a defined minimal growth medium. The Antarctic bacterium failed to grow in all commonly available minimal media [29, 37]. We have therefore developed in this study a chemically defined minimal growth medium (MM<sub>Lz</sub>) for the bacterium. It prefers medium of low osmolality (~45 mOsm) and grows proficiently in a phosphate buffer that contains ammonium, magnesium, and sulfate ions and supplemented with amino acids isoleucine and valine in addition to a carbon source (e.g., glucose or succinate). It is capable of catabolising, similar to many pseudomonads, a number of organic carbon sources including succinate, fumarate,  $\alpha$ -ketoglutarate, pyruvate, sodium citrate, sodium acetate, as well as glucose, fructose, gluconic acid, and glycerol [21, 22]. However, unlike most other pseudomonads it neither utilized mannitol nor malate. *P. syringae* Lz4W grew better in succinate and fumarate, among the tested TCA cycle intermediates. Succinate could suppress the utilization of glucose metabolizing enzymes as seen in other *Pseudomonas* species.

Utilization pattern of carbon sources were similar at both high (22 °C) and low temperature (4 °C). However, the bacterium grew faster at 22 °C and could attain maximum growth within 24 h compared to ~105 h at 4 °C. This is partly due to a difference in the lag period of growth. The lag phase is about 30 hour at 4 °C compared to 4.8 h at 22 °C in the minimal media (MM<sub>Lz</sub>) designed for *P. syringae* Lz4W. Among the twenty amino acids, all except M, W, T, G and C are used as carbon/nitrogen source. Essential requirement of two amino acids, I and V, for growth of Lz4W appears to be unique for this 'wild type' isolate of the Antarctic *P. syringae*, as *P. syringae* pv tomato DC 3000, *P. putida* or *P. fluorescence* were not reported to need any amino acid supplementation for growth in the minimal media [22, 23, 37, 38].

Auxotrophy for the two branched chain amino acids (I and V) was quite unexpected for the *P. syringae* Lz4W since it was isolated from a natural habitat of Antarctica, where source of nutrients in the soil is very scarce.

Many microorganisms when grown in enriched complex growth media might become auxotroph for certain nutrients due to the loss of biosynthetic pathway. It has been argued that the energy intensive biosynthetic steps are dispensed with by the auxotrophs when final metabolites are available. Experimentally, it has been shown that in adequate media, the auxotrophic mutants of *Bacillus subtilis* have in fact a selective advantage over prototrophic parents possibly because of energetic economy [39]. It is interesting that the natural dairy isolates of *Lactococcus lactis* subsp. *lactis* also are auxotrophic, especially for the branched chain amino acids (BCAA) leucine, isoleucine, and valine [40, 41]. Since *L. lactis* from nondairy source are generally prototrophs, it is believed that the dairy strains have originated from plant strains, which found milk as a novel rich ecological niche. In the new habitat, the dairy strain have acquired the capacity to utilize lactose via phosphotransferase system, and degrade casein by cell wall protease, but lost some features including the ability to synthesize BCAA and histidine [40, 42]. In this context, it might be interesting to note that hyperthermophilic sulfur-dependent heterotrophic Archaea from geothermal system, which grow on proteinaceous mixture, such as yeast extract, casamino acids, and purified proteins (e.g., casein and gelatin) have been found to require amino acids for growth in defined mineral medium [43, 44]. Among them, interestingly, *Pyrococcus furosius* DSM3638 requires isoleucine and valine supplementation for growth. While many of the biosynthetic operons have been found deleted in the thermophilic species [44], the auxotrophic *L. lactis* dairy strain was found to have inactivated *leu* genes via two nonsense mutations and two small deletions [40]. Just like in nutrient rich media, nutrient starvation can also lead to the loss of gene function, as shown in the case of *E. coli* population study at stationary phase [45]. Investigation of microbial evolution under conditions of prolonged starvation has led to the discovery of GASP (growth advantage in stationary phase) mutants that have the ability to grow and invade wild type majority population. Two of the GASP mutations (*rpoS* and *lrp*) arose due to gene inactivation, which changed the pattern of nutrient source utilization in favor of free amino acids that are produced by degradation of dead starved cells in the cultures [45]. In the case of Antarctic *P. syringae* Lz4W isolated from soil, it is not obvious why this bacterium is auxotrophic; it can only be surmised that *P. syringae* Lz4W might have lost or inactivated the genes for isoleucine and valine biosynthesis in response to some unknown selection pressure. Our study indicates that AHAS I and AHAS II catalyzed steps

are among the sites of inactivation in the *ilv* biosynthetic pathway.

Change of cell size of *P. syringae* Lz4W in the minimal growth medium also deserves some discussion. In a separate study we noticed that the cell size is reduced when the bacterium is grown at low (4 °C) temperature in the complex ABM [14]. Here we show that in minimal medium too *P. syringae* cell size is reduced when compared with ABM grown cells. The MM<sub>Lz</sub> grown cells look similar at both low (4°) and high (22°) temperatures without much appreciable change in size. In the case of *P. syringae*, the change in cell volume is primarily due to change in the length of cylindrical cells; at lower temperature the cells become more spherical thereby increasing the surface to volume ratio for efficient uptake of nutrients. Interestingly, the psychrotolerant bacterium *Schewanella oneidensis* MR1 and one psychrophilic *P. fluorescens* MF0 strain showed increased cell size at lower temperature of growth [38, 46]. All these results are contrary to the early belief that temperature and media composition does not affect cell size in bacteria [47].

There are many hypotheses that might explain the evolution of bacterial cell size. For example, cell size has been related to the growth rate of organisms, and these two parameters are positively correlated in batch cultures on a variety of different media [48]. In case of *E. coli*, Mongold and Lenski [49] categorically rejected the nonadaptive explanation for the increased cell size. However, it is unclear at this moment as to how larger cell size in case of *E. coli*, or smaller cell size in case of *P. syringae* under restricted nutrient conditions would be beneficial to the organisms concerned. As for the Antarctic ecosystem [50], two most significant dominating factors that shape microbial life are freezing temperature and nutrient scarcity of soil. Although the diversity of microbial life forms in the Antarctic continent has been appreciated by sequencing 16S rRNA sequences, their physiological adaptation has hardly been studied, partly due to the most common problem of culturing them in laboratory. Our ability to grow the Antarctic *P. syringae* Lz4w in a chemically defined minimal medium would be useful in this regard and perhaps to investigate the adaptive values of decreased cell size during growth at low temperature and under low nutrient condition of the minimal medium.

In conclusion, we have identified that the natural isolate of the Antarctic *P. syringae* Lz4W is auxotrophic for isoleucine and valine due to a defect in the biosynthetic pathway, and it requires these two branched chain amino acids as supplement for growth in low osmolar mineral salt medium. The chemically defined

minimal medium (MM<sub>Lz</sub>) would be useful for future study in investigating the catabolic repression control of nutrients at lower temperature of growth.

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