Microbial Production Potential of *Pantoea ananatis*

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Pantoea ananatis, a gram-negative bacterium belonging to the *Erwiniaceae* family, is a well-known phytopathogen isolated from many ecological niches and plant hosts. However, this bacterium also provides us with various beneficial characteristics, such as the growth promotion of their host plants and increased crop yield.

Pantoea ananatis | microbial production

1. Introduction

Pantoea ananatis is a gram-negative, rod-shaped, aerobic, or facultatively anaerobic bacteria belonging to the class *Gammaproteobacteria* and was recently reclassified into the family *Erwiniaceae* from *Enterobacteriaceae* [1][2]. *P. ananatis* was first described as *Erwinia ananas* by Serrano [3]. Some strains of *Enterobacter agglomerans*, *Erwinia herbicola*, and *Erwinia milletiae* that form part of the *E. herbicola–E. agglomerans* complex was assigned to the genus *Pantoea* [4]. Later, *Pantoea uredovora*, a pathogen of *Puccinia graminis*, was shown to have a high level of genomic relatedness to *P. ananas*, and the two species were synonymized [5]. *P. ananas* proposed by Mergaert et al. [5] was corrected to *P. ananatis* by Trüper and De'Clari [6]. Due to this phylogenetic history, *P. ananatis* contains various kinds of plant pathogenic strains [1]. At the same time, strains that promote plant growth that are applicable to bioremediation, or have lignocellulose degradation capacity, have been found in recent years [7].

In the field of microbial biomanufacturing, *Escherichia coli*, a model organism and a member of the family *Enterobacteriaceae*, which is a gram-negative facultative anaerobic rod, has been used industrially for the production of various substances because of its high growth rate and sugar consumption activity in the neutral pH range [8][9]. *Corynebacterium glutamicum*, a gram-positive, rod-shaped bacterium, is capable of L-glutamate fermentation [10] and has been used for the industrial production of many substances by taking advantage of its characteristic cell surface [11][12]. To date, bacterial fermentation production has been realized by mainly using *E. coli* and *C. glutamicum*, and many tools for genetic engineering have been developed for both strains [9]. However, it was clear that if both strains had the trait of robustness to pH, they would be more desirable industrial substance-producing bacteria. *P. ananatis* AJ13355 was isolated as an acidophilic bacterium [13] and has been used as an excellent host organism in previous studies to produce amino acids such as L-glutamate [14], L-cysteine [15], and isoprenoids [16][17]. The emergence of strains closely related to *E. coli* and advantageous for industrial production is important in terms of increasing the potential for substance production.

2. Characteristic of *Pantoea ananatis*

P. ananatis has been isolated from various environments and hosts and is well-known for its phytopathogenicity. *P. ananatis* causes disease symptoms in many economically important agronomic crops and forest tree species worldwide [1]. However, several strains have been known to improve the growth of plants [7], such as papaya [18], red pepper [19], sugarcane [20][21], poplar [22], and rice [23][24]. Kim et al. [19][25] determined the genome sequence of *P. ananatis* B1–9 isolated from the rhizosphere of the green onion and reported that enhanced red pepper crop yield by approximately three times and showed phosphate solubilization, sulfur oxidation, nitrogen fixation, and indole-3-acetic acid (IAA) production activities. *P. ananatis* AMG521, isolated as an endophyte from rice paddies, showed the capacity to synthesize siderophores, cellulose, and IAA, and the capacity to solubilize phosphate and increase rice yield [23]. *P. ananatis* strain 1.38, isolated from the rhizosphere of rice (*Oryza sativa* L.), has been reported to have phosphate solubilization activity, siderophore and auxin production, and cellulose, lipase, and pectinase activities [24]. It has long been known that *P. ananatis* produces carotenoids, and it has been reported that introducing the carotenoid biosynthesis genes of *P. ananatis* into a microorganism that does not produce carotenoids leads to the production of lycopene, astaxanthin, and β-carotene [26][27]. Ten complete genomes of *P. ananatis* have been determined and registered. Four were pathogenic and four had useful traits (**Table 1**).

Table 1. Complete genome of *Pantoea ananatis*.

Strain	Assembly	Chromosome Size	CDS	Plasmids	Classification	Source	Reference
Pantoea ananatis LMG 20103	GCA_000025405.2	4,703,373	4272	0	Plant pathogen	Eucalyptus	[28]
P. ananatis AJ13355	GCA_000270125.2	4,555,536	4071	2	Acidophile	Soil	[13]
P. ananatis PA13	GCA_000233595.1	4,586,378	4131	1	Plant pathogen	Rice	[29]
P. ananatis LMG 5342	GCA_000283875.1	4,605,545	4675	1	N.A.	Human	[30]
P. ananatis R100	GCA_001543055.1	4,526,803	4353	1	Antagonist against pathogen	Rice	[31]
P. ananatis YJ76	GCA_002224585.2	4,671,616	4756	3	Growth-promotion	Rice	[32]
P. ananatis PNA 97-1R	GCA_002952035.2	4,558,720	4616	2	Plant pathogen	Onion	[33]
P. ananatis NN08200	GCA_004028255.1	4,743,568	4598	2	Growth-promotion	Sugarcane	[21]

Strain	Assembly	Chromosome Size	CDS	Plasmids	Classification	Source	Reference
P. ananatis LCFJ-001	GCA_016598655.1	4,499,350	N.A.	0	N.A.	*Morus alba*	N.A.
P. ananatis TZ39	GCA_019720835.1	4,483,976	4282	2	Plant pathogen	Rice	[34]

N.A.: not applicable.

In the 1990s, researchers at Ajinomoto Co., Inc. developed L-glutamate fermentation under acidic conditions. The host needed to grow at low pH and resist high L-glutamate concentrations. In the mid-1990s, specialists from Ajinomoto Co., Inc. (Kawasaki, Japan) collected a gram-negative acidophilic bacterium from the soil of a tea plantation in Iwata City (Shizuoka, Japan), which was designated as strain AJ13355. After screening various strains for these properties, *P. ananatis* strain AJ13355 was selected as the host strain for glutamate fermentation under acidic conditions. This bacterium has proven capable of growing on various sugars and organic acids at acidic and neutral pH values and is resistant to high concentrations of L-glutamic acid [35]. The effects of pH on the specific growth rates of *C. glutamicum*, *E. coli*, and *P. ananatis* are shown in **Figure 1**.

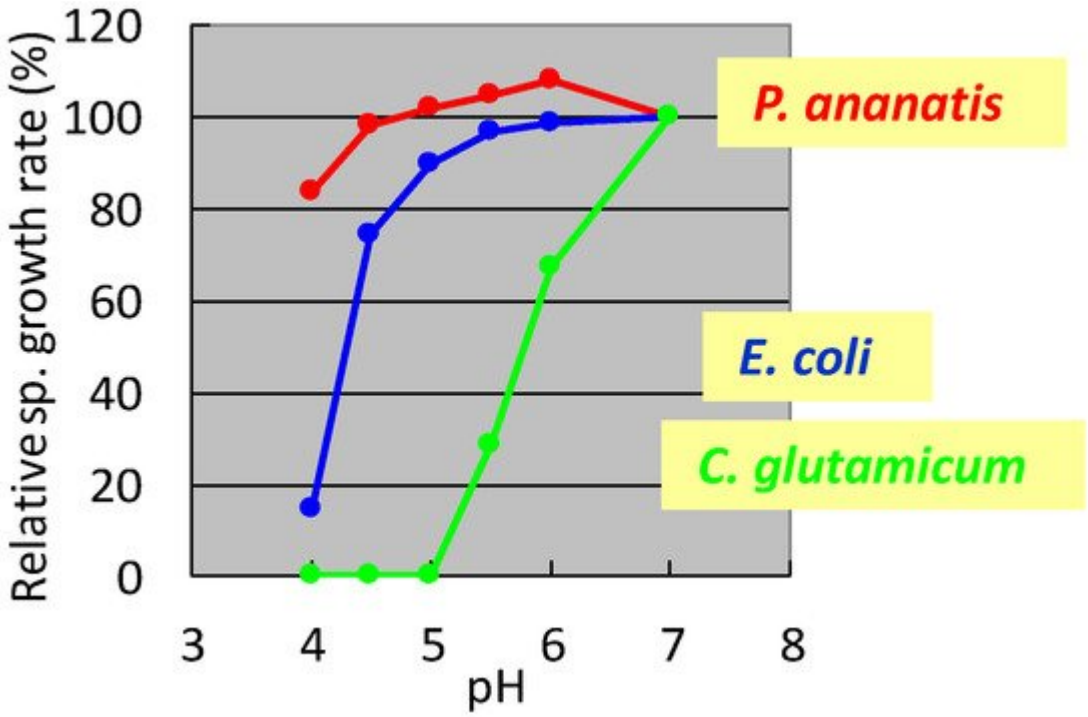

Figure 1. Effect of pH on the specific growth rate of *Corynebacterium glutamicum*, *Escherichia coli*, and *Pantoea ananatis*. The growth rate of each strain at pH 7 was 100%. *C. glutamicum*: green, *E. coli*; blue, *P. ananatis*: red. The data are typical results from three or more independent experiments.

It was shown that *C. glutamicum* showed good growth only around neutral pH, while *P. ananatis* showed better growth than *E. coli* in acidic pH. According to standard microbiological tests on its bacteriological properties and nucleotide sequencing of its 16S rRNA [36], this strain was identified as *P. ananatis* [37]. The electron micrograph of *P. ananatis* AJ13355 shows the features of gram-negative and rod-shaped bacteria (**Figure 2**).

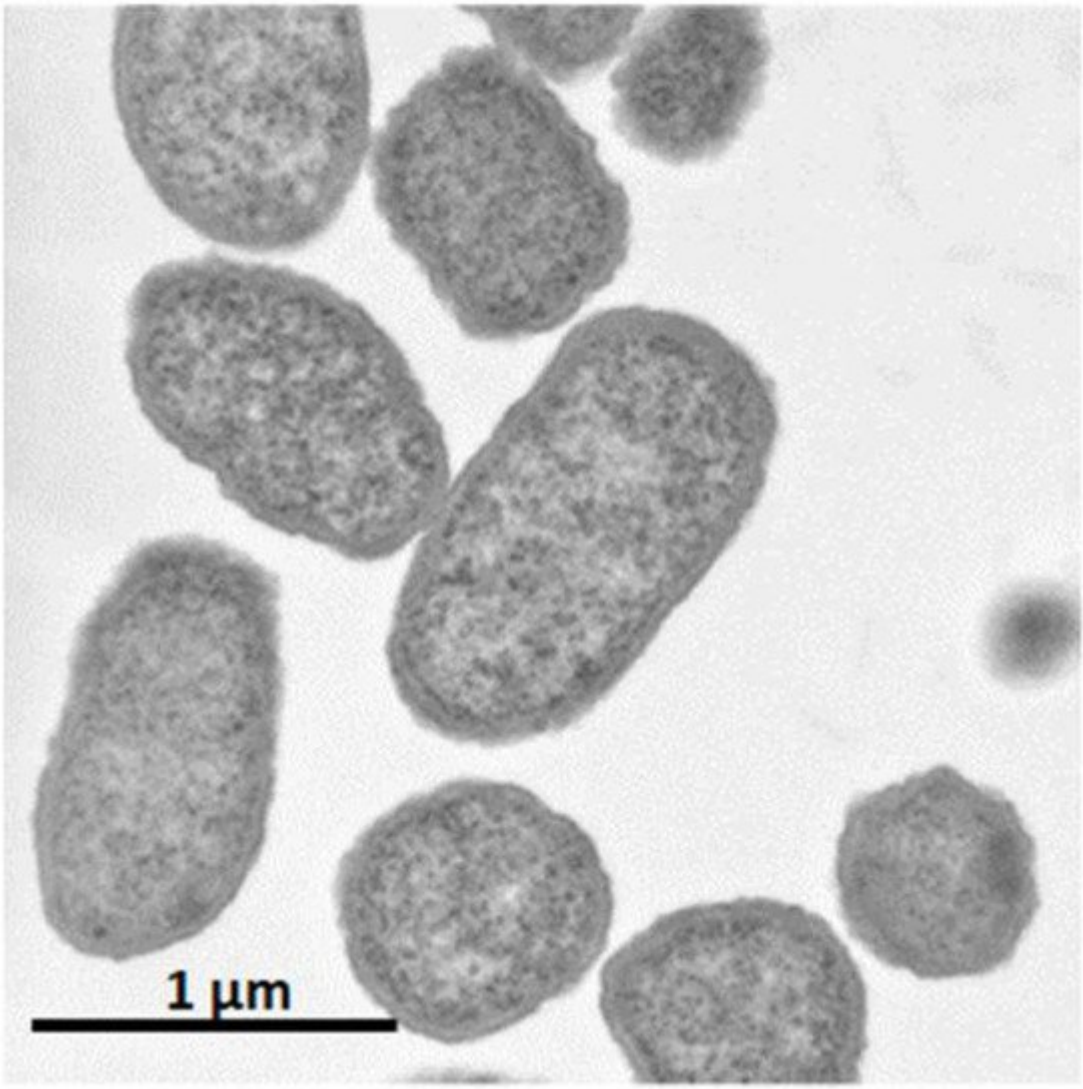

Figure 2. Electron micrograph of *Pantoea ananatis* AJ13355 at a magnification of ×30,200.

The *P. ananatis* AJ13355 strain has passed all tests required for industrialization and has been recognized as a biosafety level 1 strain. The complete genomic sequence of *P. ananatis* AJ13355 was determined and found to consist of a single circular chromosome consisting of 4,555,536 bp (DDBJ: AP012032) and a circular plasmid (pEA320) with 321,744 bp (DDBJ: AP012033). After automated annotation, 4071 protein-coding sequences were identified in the *P. ananatis* AJ13355 genome [13]. The *P. ananatis* AJ13355 genome was compared with that of *Escherichia coli*, a model organism belonging to the *Enterobacteriaceae* family to which *P. ananatis* once belonged and which was utilized in the industrial production of various substances. Short colinear regions, which are identical to the DNA sequences in the *E. coli* MG1655 chromosome, were widely dispersed along the *P. ananatis* AJ13355 genome. Conjugal gene transfer from *E. coli* to *P. ananatis*, mediated by homologous recombination between short identical sequences, has also been experimentally demonstrated [13].

To develop a general genetic tool that can be used in *P. ananatis* AJ13355, Katashkina et al. [38] constructed novel non-mobilizable derivatives of RSF1010, a well-studied broad-host-range plasmid lacking all known DNA sequences involved in the mobilization process because of the exploitation of λ Red-driven recombination between

the plasmid and an in vitro-constructed linear DNA fragment. Mobilization of the obtained RSFmob plasmid was not detected in standard tests, and high stability was demonstrated in *E. coli* and *P. ananatis*, which satisfies the biosafety requirements of genetically modified organisms used in scaled-up production [38].

To develop highly active producer strains with metabolically engineered pathways, it is necessary to manipulate many genes and express them individually at different levels or under separate regulatory controls. The construction of plasmid-less marker-less strains using sequential chromosome modifications, including deletions and integration of genetic material, has many advantages for further practical exploitation of these bacteria in industry. The λ Red-recombineering technique previously developed in *E. coli* is a high-performance tool for the rapid construction of precise genome modifications, such as deletion or insertion of genetic material, nucleotide changes, modification of regulatory regions, and construction of unmarked mutations. Although the expression of λ Red genes in *P. ananatis* was highly toxic, a mutant strain, SC17(0), grew well under simultaneous expression of λ *gam*, *bet*, and *exo* genes. Using this strain, site-specific and homologous recombination of phage λ, together with the possible transfer of marked mutations by electroporation of the chromosomal DNA, were adapted to genetically engineer *P. ananatis* AJ13355 and its derivatives [39]. Minaeva et al. [40] developed a new method of foreign DNA insertion for the step-by-step construction of plasmid-less marker-less recombinant *E. coli* strains with a chromosome structure designed in advance. The dual-in/out strategy is based on the initial Red-driven insertion of artificial φ80-attB sites into the desired points of the chromosome followed by two site-specific recombination processes: first, the φ80 system is used for integration of the recombinant DNA based on selective marker-carrier conditionally replicated plasmid with φ80-attP-site; and, second, the λ system is used for excision of the inserted vector part, including the plasmid ori-replication and the marker, flanked by λ-attL/R-sites [40]. Andreeva et al. [41] adopted a dual-in/out recombineering-driven strategy to integrate DNA fragments into targeted points on the *E. coli* chromosome for application in *P. ananatis*. *P. ananatis pqqABCDEF* was cloned in vivo and integrated into the chromosomes of *P. ananatis* using the dual-in/out strategy. The introduction of a second copy of *pqqABCDEF* to *P. ananatis* SC17(0) doubled the accumulation of PQQ [41]. This new approach has facilitated the design of recombinant marker-less and plasmid-less strains. It allows for the inclusion of large artificial inserts that are difficult to introduce using commonly used PCR-based recombination procedures. Katashkina et al. [42] further improved the dual-in/out system to a method that simultaneously incorporates a few DNA fragments into specific loci on the genome. They divided the mevalonic acid pathway into acetyl-CoA to mevalonic acid (MVA), MVA to phosphomevalonate, and the remaining pathway (see Isoprenoid production). Through the improved dual-in/out system and electroporation efficiency in *P. ananatis*, they were able to handle plasmids for all three parts simultaneously. They showed that their experimental design was sufficient to remove all selection markers [42]. Consequently, it is now possible to evaluate the production of genetically engineered strains that retain the desired multiple genetic traits, resulting in an increase in breeding speed.

4. L-Cysteine Production

L-Cysteine is an important amino acid with many applications in various industries, including pharmaceuticals, food, and cosmetics. Bacterial fermentation is well-established for many major L-amino acids in commercial

production; however, L-cysteine is one of the few exceptions, for which fermentative production methods have been demonstrated only in recent years and are still under development [43][44]. Because of the cytotoxicity of L-cysteine, bacterial cells are equipped with several modes of stringent metabolic regulation that allow them to strictly control the intracellular levels of L-cysteine, making overproduction of this compound more challenging. Dissecting these multifaceted and complicated intracellular regulations and freeing them from negative feedback controls is essential to achieve efficient overproduction. These regulatory mechanisms include feedback inhibition of two key enzymes, serine acetyltransferase (SAT) and 3-phosphoglycerate dehydrogenase (3-PGDH) by L-cysteine and L-serine, respectively, along with the L-cysteine biosynthetic pathway. CysB, a master regulator of sulfur assimilation and L-cysteine metabolism, controls the expression of most of the biosynthetic and metabolic genes associated with L-cysteine at the transcriptional level (**Figure 3**).

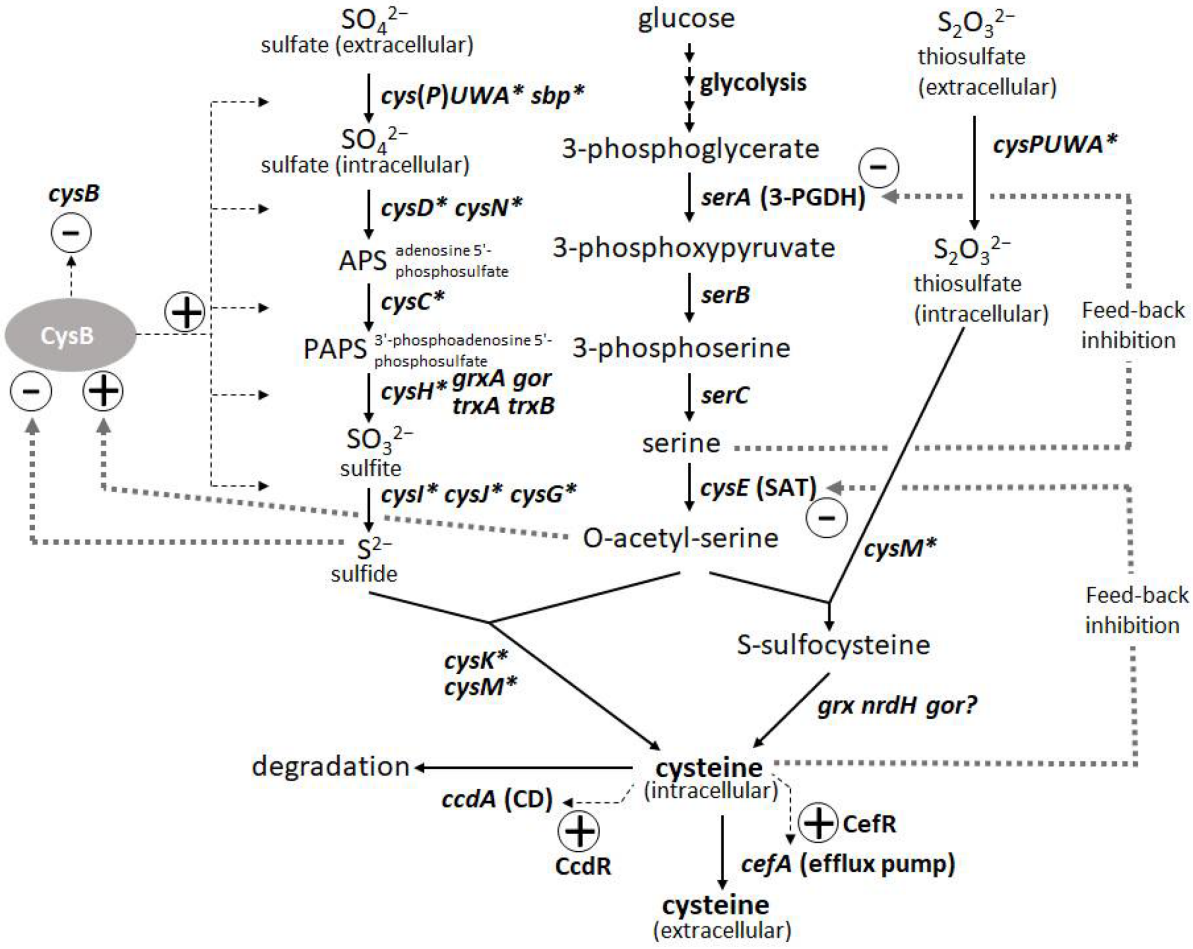

Figure 3. Biosynthetic pathway and regulation of L-cysteine in *Pantoea ananatis* AJ13355 [45]. Thin and thick dotted lines indicate transcription and protein regulation, respectively. Asterisk indicates CysB regulon.

The degradation of L-cysteine provides another mode of regulation wherein cysteine desulfhydrases (CDs) play a central role in protecting bacterial cells from intracellular over-accumulation. The efflux systems of L-cysteine by specific exporters add an additional layer of regulation, in which they work as a safety valve to cope with the rapid increase in its intracellular levels. Thus, L-cysteine levels are regulated by its biosynthesis, degradation, and efflux [45]. To achieve overproduction, negative regulations, namely SAT and 3-PGDH, must be deregulated, and positive

regulations, namely CysB regulons of biosynthesis, must be enhanced. CDs have to be removed, but they must be combined with the enhancement of efflux transporters because the transporters contribute to recovering damaged cells by accumulating L-cysteine under deficient conditions of CDs and promoting extracellular production of L-cysteine as its oxidized form L-cystine (L-cysteine can be easily oxidized extracellularly during aerobic cultivation). Once these core factors specific to L-cysteine production are regulated, general approaches such as enhancing bottlenecks and their biosynthetic pathways and shutting down the pathways to byproducts become more effective [15]. To date, among many amino-acid-producing microbes, *P. ananatis* and *E. coli* are advanced bacterial hosts for L-cysteine production [15][46].

The introduction of mutated key biosynthetic enzymes that are free from feedback inhibition is the first step in the development of amino-acid-producing microbes. There are two key enzymes in the L-cysteine biosynthetic pathway in *P. ananatis*: SAT and 3-PGDH encoded by *cysE* and *serA*, respectively. Mutations that remove the feedback inhibition by L-cysteine have been well-studied and applied to L-cysteine production in *E. coli* (**Figure 3**). Effective mutations identified for 3-PGDH in *E. coli* [47] could apply to the corresponding position of this enzyme in *P. ananatis* [48]. Kai et al. [49] identified many substantial effective mutations in the SAT of *E. coli* and *P. ananatis* that abolished feedback inhibition by comparing the crystal structures of SAT with and without the allosteric inhibitor. The basic preliminary L-cysteine-producing strains can be constructed using these mutated enzymes, which typically produce traces of L-cysteine in productive media.

The second major step in genetic engineering is identifying and removing the genes involved in L-cysteine degradation. In *E. coli*, at least five CDs (TnaA, MetC, MalY, CysK, and CysM [50][51]) and one cysteine desulfidase (YhaM [52][53]) have been identified and demonstrated to exert positive effects on L-cysteine production through gene deletion. While *E. coli* has developed rather complicated degradation mechanisms involving multiple degradation enzymes, *P. ananatis* possesses the only CD encoded by *ccdA*. CcdA in *P. ananatis* is the major and strongest CD that is induced intensively by L-cysteine using CcdR encoded adjacent to *ccdA* in the corresponding locus; therefore, this CD is proposed to have a more distinctive function in L-cysteine decomposition to cope with the sudden increase in both intracellular and extracellular L-cysteine levels [54]. Deleting *ccdA* in *P. ananatis* is very effective for producing L-cysteine, as it removes detectable levels of CD activity in cells [15][45]. *P. ananatis* may have advantages over *E. coli* in managing degradation activity. *E. coli* strains with multiple gene knockouts could still exhibit significant CD activity [51], indicating that there were additional unidentified CDs that presumably negatively affected L-cysteine production. Moreover, many of the CDs in *E. coli* have significant metabolic functions (e.g., TnaA is a tryptophanase, and CysM and CysK are cysteine synthases), whose deletion may affect essential physiological functions, including growth and biosynthesis of L-cysteine.

The third step was to identify and utilize the efflux system of L-cysteine. Because of the deletion of CD gene(s), the cells exhibited poor growth that was due to the increased intracellular levels of L-cysteine. The introduction of efflux pumps increases the production of L-cysteine and recovers the damaged cells from poor growth caused by accumulated L-cysteine. YdeD and YfiK are functional efflux pumps in *E. coli* for L-cysteine [54][55]. However, since they were identified while searching for factors effective for overproducing L-cysteine, their substrates and physiological function in L-cysteine metabolism and regulation are unclear. However, *P. ananatis* has developed

more specific efflux pumps of L-cysteine, associated with its metabolism and regulation. CefA and CefB of *P. ananatis* were discovered by screening for factors that confer resistance to high concentrations of L-cysteine (**Figure 3**). CefA was found to be inducible by L-cysteine using its counterpart regulator CefR, encoded adjacent to *cefA* in the corresponding locus; therefore, it has been proposed that it functions as a specific safety valve of L-cysteine [45]. Both CefA and CefB were demonstrated to be involved in L-cysteine overproduction in *P. ananatis*, where they significantly contributed to high-level production and recovery from L-cysteine toxicity caused by the absence of its decomposer *ccdA* [15]. *P. ananatis* may have an advantage over *E. coli* in exporting L-cysteine because it possesses more specific and efficient efflux systems that may allow the export of L-cysteine specifically without leaking out some essential metabolites required for cell viability.

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