Heterologous Protein Production in *P. fluorescens*

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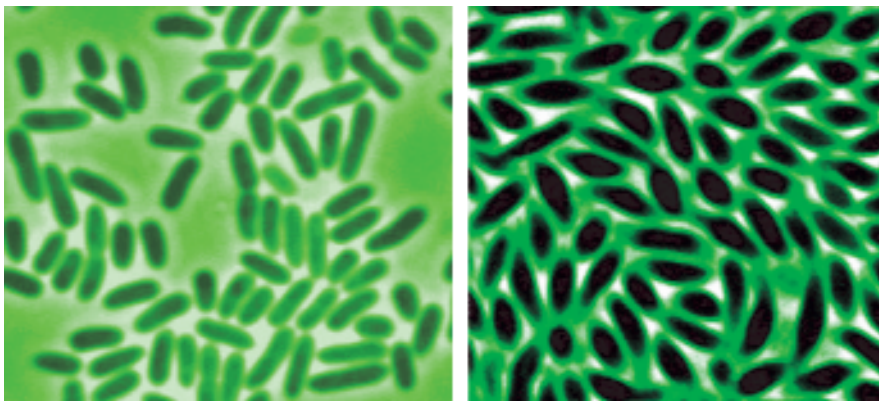
Rapid expansion in the biotechnology and biopharmaceutical industries during the past few years has necessitated the expression of a spectrum of recombinant proteins in different host systems for a number of purposes. In some applications, a large number of distinct proteins are needed in small quantities for screening applications or structural determinations. In other cases, quantities approaching metric-ton scales are needed for specific therapeutic applications. Most therapeutic proteins have been produced in either mammalian cell culture systems — Chinese hamster ovary (CHO) cells are the most common — or in microbial systems, with *Escherichia coli* as the most common microbial host.

Alternative expression systems are also being used, developed, and evaluated. They include other mammalian and insect cell lines, yeasts and other fungi, and bacterial host cell systems. Each offers specific system advantages, but it is unclear which will ultimately be the most useful for the range of applications in therapeutic protein production. In fact, several systems are likely to reach broad acceptance for particular niches in protein production.



Before induction with IPTG, cells of *P. fluorescens* appear as normal Gram-negative rods (left). Following induction (right), the cells fill up with recombinant protein that can accumulate to more than 25 g/L in a fermentation. DOWPHARMA AND BIOTECHNOLOGY R&D (WWW.DOWPHARMA.COM)

Efficient discovery of efficacious new protein pharmaceuticals is essential if medicine is to meet future challenges in treating disease. However, the ability to manufacture pharmaceuticals economically is increasing in importance as drug development costs escalate. New production technologies must yield high titers of protein in active form. Many proteins in development today are monoclonal antibodies (MAbs), antibody fragments, or antibody conjugates that present high dosage demands or special production challenges related to recovery of active material.

CORE EXPRESSION PLATFORM

The current platform for recombinant protein production in *Pseudomonas fluorescens* involves a family of host strains derived from *P. fluorescens* biovar I (MB101) and stable, self-replicable, but nonconjugative expression plasmids of varying copy number. These host strains are amenable to genetic or molecular manipulations and can be cultivated at high cell densities.

As with other pseudomonads (1, 2), *P. fluorescens* can be cultivated to high densities in bioreactors using a simple but balanced, defined mineral salts medium supplemented with a carbon source (e.g., glucose) and an inorganic nitrogen source (e.g., ammonia). The organism is a strict aerobe, so adequate oxygen transfer to the culture is necessary for optimal growth. Nevertheless, from our experience, maintaining

PRODUCT FOCUS: PROTEINS AND PEPTIDES

PROCESS FOCUS: PRODUCTION

WHO SHOULD READ: PROCESS DEVELOPMENT, MANUFACTURING

KEYWORDS: MICROBIAL EXPRESSION, FERMENTATION, *PSEUDOMONAS*, GENETIC ENGINEERING

LEVEL: INTERMEDIATE

dissolved oxygen above a certain level in the bioreactor does not appear to be as critical for *P. fluorescens* as for *E. coli*. Also unlike *E. coli, P. fluorescens* does not accumulate acetate during fermentation. As with other pseudomonads, its glucose uptake is preferentially through the oxidative rather than the phosphorylative pathway (**3**, **4**). With optimal fermentation conditions and carbon feeding, biomass levels of greater than 100 g/L dry weight — accompanied by recombinant expression levels at >50% of total cell protein — can be achieved with *P. fluorescens* at >10,000-L production scales without the need for oxygen supplementation.

In this system, expression of heterologous genes is driven by transcription promoters of varying strengths, such as the *tac* and *lac*UV5 promoters derived from *E. coli* sequences. Several *P. fluorescens* endogenous promoters have been developed for recombinant protein expression. Optimal translation initiation signals and strong transcription terminators are also available.

Prokaryotic microorganisms such as *P. fluorescens* can secrete proteins to their extracellular medium or to an intracellular compartment (the periplasmic space) that lies between the inner and outer membranes. Secretion can be of value in decreasing the costs of protein recovery. The biochemical apparatus that forms protein disulfide bonds in prokaryotes lies within the periplasmic space because the cytoplasm, where protein synthesis occurs, is too reduced to allow those bonds to form chemically. Certain genetically modified strains of *E. coli* will form disulfides intracellularly (**5**). Otherwise, all disulfide-bonded proteins in prokaryotes are found in the periplasmic space or are secreted outside of the cell entirely.

Many mammalian-derived proteins of therapeutic interest, including MAbs and some engineered antibody fragments, contain disulfide bonds. When produced microbially, therefore, they should be secreted at least to the periplasmic space if formed de novo in an active state (**6**). Secretion is typically mediated by a short, 20–40 amino acid leader sequence on the amino terminus of secreted proteins. That sequence is specifically removed at the periplasmic face of the inner membrane by a signal peptidase. Capable of efficiently directing secretion of heterologous proteins to the periplasm of *P. fluorescens,* such sequences have been discovered through combined genomic and functional means.

Of several signal sequences identified, the signal from phosphate binding protein (*pbp*) in particular has been found effective at efficiently transporting single-chain antibodies and other mammalian-derived proteins. In addition, quantitative removal of this secretion leader from a number of different recombinant proteins has been demonstrated, and secretion led to the recovery of disulfide-bonded proteins in their native, active conformations. Recent evidence suggests that osmotic shock or other extraction techniques can effectively release protein from the periplasm at commercially relevant scales (**7**, **8**).

Antibiotic-Free Plasmid Maintenance

In traditional microbial heterologous protein production, antibiotic-resistance genes are essential to maintaining the genetically engineered plasmids in their correct form. Such genes can cause a problem if they aren't completely removed from the final product. Regulatory agencies discourage residual antibiotic resistance coding gene DNA in drug products because of a perceived risk for genetic transfer to the intestinal flora of humans. In some instances, additional expensive processing steps are needed to degrade or remove that DNA so it does not contaminate the final product.

The antibiotics themselves used for plasmid maintenance can raise regulatory concerns as well. This is particularly true in biocatalysis used to create drug intermediates — or in food uses for enzymes, where catalyst preparations typically are not as pure as protein pharmaceuticals must be.

Removing the antibiotic resistance genes from production strains would eliminate those issues. To that end, we replaced antibiotic resistance genes on the plasmids with others encoding essential proteins for steps in intermediary metabolism. The corresponding auxotrophic *Pseudomonas* strains were made by deleting corresponding genes in the chromosome. Two genes were adapted for this purpose: *pyrF* (encoding orotidine 5′-decarboxylase, an essential step in biosynthesis of uracil) and *proC* (encoding pyrroline-5-carboxylate reductase, the last step in proline biosynthesis). These open reading frames (ORFs) were amplified using polymerase chain reaction (PCR) and then cloned in place of the antibiotic-resistance genes on two plasmids with the auxotrophic selectable markers. When

Figure 1: Expression of a single chain antibody (scFv) in *P. fluorescens*. Cells were grown in 20-L fermentors with a defined, mineral salts medium. The promoter sequence was *tac,* and the genes were induced with isopropyl-thiogalactopyranoside (IPTG) to express the scFv for a total of 24 hours before harvest and preparation of this gel. Samples were loaded by a constant biomass technique. "Sol" and "Ins" refer to the soluble and insoluble protein fractions separated by centrifugation of the crude cell lysate. Amount of specific protein was determined by densitometry against a standard loaded on the same gel (not shown).

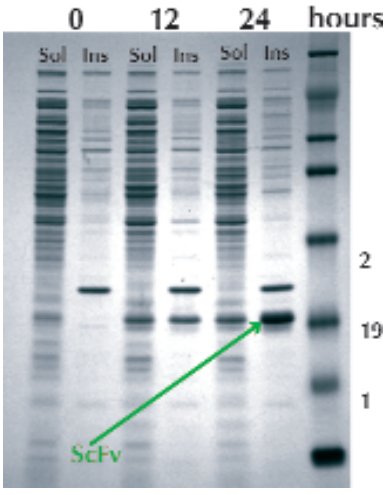
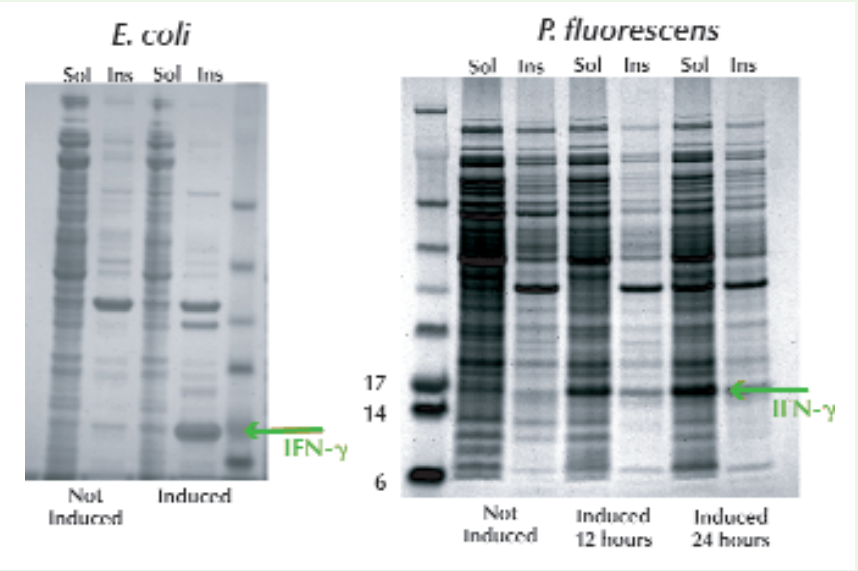


Figure 2: Conditions as described in the first figure for *P. fluorescens*. The *E. coli* cells were induced for four hours using IPTG, and the promoter sequence used was T7. Samples were loaded by a constant biomass method. Amount of specific protein was determined by densitometry against a standard loaded on the same gel (not shown).



compared (in 20-L fermentations) with strains harboring the antibiotic resistance coding DNA, the production of target proteins in the strains carrying the auxotrophic markers was identical. Plasmids maintained through complementation have been shown to be as stable as those maintained by antibiotic resistance selection (9).

Genomics and Functional Genomics

We determined the genome sequence of *P. fluorescens* strain MB214 — a derivative of MB101 biovar 1 (10) with a chromosomal insertion of the *E. coli lac* operon — while developing it into a production platform strain for the manufacture of proteins, peptides, and metabolites. Knowledge of the entire genome allows rapid, directed changes to individual genes or groups of genes, thus enabling fast, sophisticated pathway engineering and enhancement of specific gene expression. *P. fluorescens* is easy to manipulate genetically, which enhances our engineering capabilities. The 6.5-megabase genome of strain MB214 is similar in size to other pseudomonads whose sequences have already been established (11–13). More than 6200 ORFs were identified using gene finder tools, and gene functions were assigned using standard software.

Reconstruction of the *P. fluorescens* metabolism based on the annotated genes reveals that it is a metabolically versatile organism. More than 700 pathways were identified containing numerous (sometimes redundant) pathways within this group. Despite a high level of genome conservation in the pathogenic *P. aeruginosa* PAO1, key virulence factors (e.g., exotoxin A and PrpL proteinase) are absent from the established genome sequence of *P. fluorescens* strain MB214. Other *P. fluorescens* strains whose genomes have been sequenced — Pf0-1 (14) and SBW25 (15) — are also missing those factors.

Bioinformatics analyses of the MB214 genome to uncover protein secretion systems indicated that all known microbial protein export systems are present in MB214 except for one: the type IV secretion system. That one is commonly plasmid borne, and no plasmids have been found in MB214. Comparison of the genomes of *P. aeruginosa* PAO1 with the two *P. fluorescens* strains MB214 and Pf0-1 revealed that the same protein secretion systems are present in all three pseudomonads, differing only in the number of paralogous exporters (16). The presence of multiple protein secretion systems opens avenues toward genetically engineering production strains that can secrete desired protein products into their subcellular periplasmic spaces or into their growth media for easy and cost-effective protein recovery.

Knowledge of the microbial genome sequence also allows establishment of functional genomics capabilities such as DNA microarray and proteomics for gene and protein expression analysis. These capabilities are very useful for monitoring cellular metabolic conditions during the growth and protein production phases of fermentation. Transcriptional profiling and proteomics technologies are analytical tools for learning about host cells' metabolic states as well as the temporal profile of desired gene transcripts and protein production during fermentation experiments. Proteomics can further supply information about the yield and stability of overproduced protein as well as the processing sites of signal sequences that target it into the periplasm. Additionally, proteomics can help direct protein purification processes by identifying copurifying proteins. That could lead to altering current purification processes or aid in steering directed genetic engineering to eliminate interfering proteins. Together with other tools, these capabilities will allow refined analysis and modification of both fermentation and protein expression in *P. fluorescens*.

Protein Production

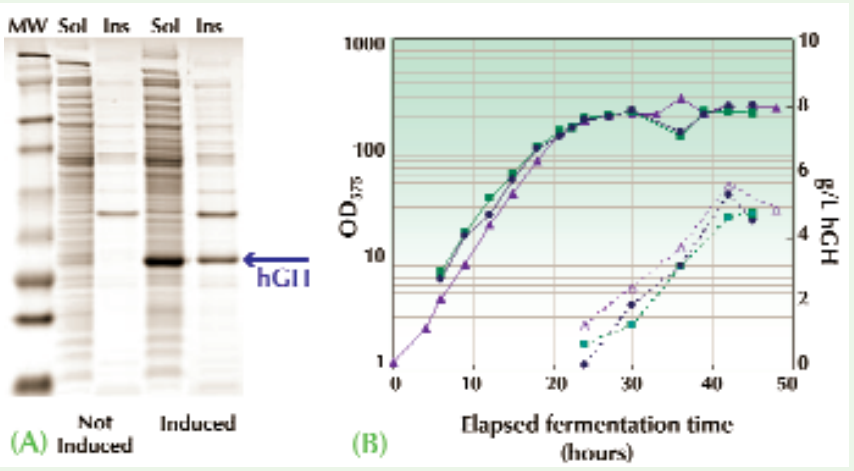
MAbs and engineered antibody fragments represent a large percentage of therapeutic and diagnostic proteins currently in development. One anti-ß-galactosidase single chain antibody, Gal2 (17, 18) containing two protein disulfide bonds, was secreted to the periplasm of *P. fluorescens*. Figure 1 is a sodium dodecyl sulfate polyacrylamide gel electrophoretogram (SDS PAGE) showing that the *pbp–gal2* fusion was expressed in *P. fluorescens* to 3 g/L total (soluble and insoluble) of fully processed protein at the 20-L scale. Twelve hours after induction, the same amount of Gal2

protein was present in the soluble and insoluble cellular protein fractions. But the insoluble fraction had increased to at least 66% of the total by 24 hours post-induction, suggesting that some cellular protein folding function had become rate limiting by that time. Purified protein was found to be active in an anti-ß-galactosidase ELISA (enzyme-linked immunosorbent assay).

Interferon-γ is a protein pharmaceutical that, when expressed in *E. coli*, can be formed as insoluble and inactive protein. It has been difficult to renature recovered insoluble protein to its native state (**19**). The experiment described in Figure 2 (SDS PAGE) compares expression of interferon-γ by *E. coli* with that of *P. fluorescens*. As expected, the interferon-γ recovered from a 20-L *E. coli* fermentation (about 2 g/L) was insoluble. In that experiment, we used the bacteriophage T7 promoter (**20**) to drive interferon-γ expression. However, we found that interferon-γ recovered from the 20-L *P. fluorescens* fermentation was nearly all in the soluble fraction and expressed at about 4 g/L total. The increased overall yield and dramatically improved expression of soluble and active protein seen in *P. fluorescens* clearly highlights the advantages it can offer in producing recombinant proteins. Protein purified from the soluble fraction was found to be as active as a commercial standard in the typical in vitro viral growth inhibition assay used for interferon-γ. Protein was recovered from the soluble fraction at 95% purity in two ion-exchange chromatography steps.

Pharmaceutical-grade recombinant human growth hormone (hGH), which contains two disulfide bonds in its native state, is currently produced in *E. coli* cytoplasmically as inclusion bodies that can be readily refolded to the active state (**21**). It would be an advantage, however, if this protein could be produced in a soluble and active form. We expressed hGH in *P. fluorescens* as a secreted protein using the *pbp* secretion leader referred to above. Panel A of Figure 3 is a photograph of an SDS PAGE showing that the hGH produced was found in the soluble cellular fraction at more than 5 g/L. In addition, the *pbp* sequence was completely and precisely cleaved from the native hGH sequence. Panel B of Figure 3 shows the reproducibility of the 20-L fermentation used to produce recombinant hGH. Three separate fermentations were similar in the progress of their growth phases, in fermentation performance following induction, and in the quantity of product made.

Figure 3: Conditions as described in the first figure. Cells were induced for 24 hours with IPTG. (A) Samples were loaded by a constant biomass method. Amount of specific protein was determined by densitometry against a standard loaded on the same gel (not shown). Blue arrow indicates the location of hGH. (B) Three 20-L fermentations were used to generate samples on the Coomassie-stained gel. Cells were grown at 32 °C in a mineral salts medium with glucose as the carbon source, then induced with IPTG 24 hours into the fermentation.



A Viable Alternative

Rapid, efficient expression of therapeutic molecules is needed to manage production costs and provide effective, affordable products — especially for higher-volume applications. With that in mind the *P. fluorescens* strain described in this paper has many favorable properties. The organism is grown in a completely defined mineral salts medium, with no added animal components or organic nitrogen of any kind. Our fed-batch fermentation process is well characterized, and scale-up is predictable and rapid to thousands of liters. Cell densities >100 g/L of dry cell weight are routinely achieved in standard fermentation vessels without oxygen supplementation. The organism is unusually well suited for high-level expression and will tolerate a wide range of fermentation conditions. Recovery and downstream purification procedures are standard and consistent with those used for molecules expressed by *E. coli*.

Knowledge of the *P. fluorescens* genome sequence enables rapid strain engineering to improve gene expression as well. Established protein expression techniques used during fermentation experiments can be monitored by functional genomic tools to gauge the metabolic state of host cells and to provide temporal profiles of desired gene transcripts and protein production. Resulting data can be used to further improve strain performance through directed genetic changes.

Extensive pathogenicity and toxicology studies have shown *P. fluorescens* to be safe for therapeutics production (**10**). Work continues to add data relevant for future regulatory submissions. High-volumetric and specific expression of a range of therapeutic molecules as soluble, active, and secreted products makes *P. fluorescens* a compelling alternative for the microbial expression of biologicals for human health.

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