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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Development of a fermentation process based on a defined medium for the production of pregallidermin, a nontoxic precursor of the lantibiotic gallidermin

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Abstract In this work, a defined medium was developed and optimized for the mutant strain *Staphylococcus gallinarum* *ΔP*, which produces pregallidermin (PGDM), a nontoxic precursor of the lantibiotic gallidermin (GDM). The availability of a defined medium is a prerequisite for a rational process development and the investigation of medium effects on final product concentration, yield, and volumetric productivity. We identified four vitamins and three metal ions as essential for growth and PGDM production with *S. gallinarum ΔP*. The strain was capable of growing without any added amino acids, but the addition of proline had a strong growth-stimulatory effect. The concentrations of all essential compounds were balanced in a continuous culture using a medium-shift technique. Based on this balanced medium, a fed-batch process was developed in which *S. gallinarum ΔP* was grown up to a biomass concentration of 67 g l^{-1} and produced 1.95 g l^{-1} PGDM, equivalent to 0.57 mM. In the fermentation broth, we identified other GDM precursors in addition to those with a 12 or 14-amino-acid-long leader peptide that had been observed previously. Including those precursors with shorter leader sequences, the final concentration would correspond to 0.69 mM. In molar terms, this represents a roughly fourfold or fivefold increase, respectively, over established, complex medium-based gallidermin production processes (Kempf et al. 2000). With the same medium and feed protocol, the maximum concentration of mature GDM produced by wild-type *S. gallinarum* Tü 3928 was only 0.08 mM.

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Introduction

Infectious diseases is the second leading cause of death worldwide (Projan 2003), and nowadays 70% of hospital-acquired infections in the USA are resistant to one antibiotic or more (Leeb 2004). Therefore, an impelling need for new drugs is emerging to combat the current generation of multidrug-resistant pathogens. Lantibiotics are posttranslationally modified antimicrobial peptides (McAuliffe et al. 2001) with great potential for the treatment of infections provoked by problematic Gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci (Deves-Broughton et al. 1996; Krusewska et al. 2004; Breukink and de Kruijff 2006). However, due to the lack of a platform permitting their large-scale manufacturing in a cost-effective manner, so far, no lantibiotic has been marketed as a clinical product (Hancock and Sahl 2006).

Gallidermin (GDM) is a type A lantibiotic naturally produced by *Staphylococcus gallinarum* Tü 3928 (Kellner et al. 1988; Schnell et al. 1988; Schnell et al. 1989; Kempf et al. 2001) that possesses high bactericidal activity and low cytotoxicity (Kellner et al. 1988; Maher and McClean 2006).

Maximum product concentrations in GDM fermentations have been limited to approximately 0.14 mM (300 mg l^{-1}; Ungermann et al. 1991; Kempf et al. 1997, 1999a, b), which is in the same range of a few hundred milligrams per liter reported for the similar epidermin (Hörner et al. 1989; Hörner et al. 1990; Ungermann et al. 1991) and other lantibiotics such as nisin (de Vuyst and

Vandamme 1992, 1993; de Vuyst 1994; Parente and Ricciardi 1999) and Pep5 (Hörner et al. 1989, 1990). The most likely explanation for these low titers is product inhibition. In fact, wild-type (wt) *S. gallinarum* was inhibited by GDM concentrations above 0.10 mM (225 mg l^{-1}; Ungermann 1992), and cultivation protocols involving in situ product removal yielded substantially higher amounts of totally produced gallidermin per liter bioactive volume (Ungermann et al. 1991).

Previously, we developed a novel two-stage production strategy, where the protease GdmP-deficient strain *S. gallinarum ΔP* fails to cleave off the GDM leader sequence entirely and hence produces a GDM precursor called pregallidermin (PGDM; Valsesia et al. 2007). This precursor consists of the fully modified GDM extended by a 12–14-bp leader sequence. Even though this extension is shorter than predicted from the gene sequence, it is sufficient to prevent the toxic effect of GDM. After fermentation, PGDM can be easily converted to GDM by tryptic digestion (Valsesia et al. 2007). The development of a high cell density culture protocol for *S. gallinarum ΔP* allowed a PGDM titer up to 0.35 mM. However, a very high cell dry weight (CDW) concentration of 120 g l^{-1} was necessary to obtain this concentration (Medaglia et al. 2010). Such a high cell density is expensive to achieve, and the required large amounts of complex medium components (e.g., yeast extract) would make the purification of (P)GDM from the cultivation broth more difficult.

To circumvent the problems associated to the high cell density protocol, an increase in specific productivity is required. As genetic engineering for the construction of a PGDM overproducer is difficult (Valsesia 2008), we investigated the medium composition. So far, all production media for GDM and PGDM fermentations were complex, and the highest GDM production titers were obtained with a medium containing yeast extract, maltose, and $CaCl_2$ (Kempf 1999a, b). In fact, it is established that critical components of the medium can regulate the synthesis of the compound of interest (Masurekar 2008), and both stimulatory as well as inhibitory effects can occur. Specific amino acids were described to stimulate GDM production (Ungermann 1992; Kempf 1999a), but high concentrations of glucose and phosphate led to decreased GDM and Pep5 production in *S. gallinarum* and *Staphylococcus epidermidis* batch cultivations (Hörner et al. 1990).

Some defined media for the cultivation of staphylococcal strains have been reported, where several amino acids, vitamins, and metal ions were required (Gould and Lennarz 1970; Wu and Bergdoll 1971; Miller and Fung 1973; Emmett and Kloos 1975; Cove and Holland 1980). Specifically, for *S. gallinarum*, a defined medium for the production of GDM containing 17 amino acids, ten metal ions, and nine vitamins has been developed (Ungermann 1992). Critically, it required bovine serum albumin (BSA) for GDM production, which is rather costly and does not allow the operation of an economic process. Here, we develop a defined medium that is optimized for *S. gallinarum ΔP* biomass yield and results to be highly effective for PGDM production.

Material and methods

Bacterial strains, chemicals, and media

S. gallinarum Tü 3928 (DSM4616) and *S. gallinarum ΔP* (DSM17239) were grown and stored as described elsewhere (Valsesia et al. 2007). The latter strain carries a *gdmP* gene that is disrupted by a kanamycin resistance gene. All chemicals were either from Sigma Aldrich (Buchs, Switzerland) or Roth (Reinach, Switzerland) unless stated otherwise. Stock solutions for KH_2PO_4, $Na_2HPO_4\times 2\ H_2O$, NaCl and $(NH_4)_2SO_4$, $MgSO_4$, $CaCl_2$, maltose, antifoam industrol 204 (BASF, Mount Olive, NJ, USA), and 3-(*N*-morpholino) propanesulfonic acid (MOPS) were separately autoclaved. Trace element solution US* (Panke et al. 1999), vitamins, amino acids, and kanamycin stock solutions were sterile-filtered.

Determination of nutritional requirements in shake flasks

Experiments towards the determination of the nutrimental requirements were based on medium sGM1 (Table 1), to which different combinations of amino acids were added (see results). After addition, the amino acid concentrations in the medium were (in milligram per liter) L-Ala 15, L-Arg 12.5, L-Asn 5, L-Asp 25, L-Cys 0.5, L-Glu 79.5, L-Gln 5, Gly 7, L-His 10, L-Ile 20, L-Leu 25, L-Lys 5, L-Met 7, L-Phe 20, L-Pro 40, L-Ser 10, L-Thr 8.5, L-Trp 2, L-Tyr 2, L-Val 28. These concentrations correspond to the concentrations in a 5 g l^{-1} casamino acids solution (as specified by the vendor, Becton Dickinson, Sparks, MD, USA). Where mentioned, single or multiple components were omitted from the preparation, giving rise to medium variants. For the growth experiments, *S. gallinarum ΔP* was inoculated from an agar plate into 3 ml precultures of medium sGM1 and cultivated for 12–14 h at 37 °C and 190 rpm. Then, precultures were centrifuged at 13,200 g for 2 min at room temperature and the cells were washed with 2×8 ml of washing solution (2% (*w*/*v*) aqueous NaCl). Finally, the pellet was resuspended in 4 ml of the washing solution and diluted 100-fold in fresh media sGM1 with varying additions and incubated at 37 °C and 190 rpm. The maximum specific growth rates (μ_{max}) were determined by fitting at least three data points from the exponential growth phase in a log-linear plot by linear regression.

Table 1 Media compositions for cultivation and feed media for *S. gallinarum ΔP*

Compound (unit)	sGM1	sGM2	fGM2
Maltose (g l^{-1})	10.0	20.0	333
KH_2PO_4 (g l^{-1})	8.50	1.60	35.0
Na_2HPO_4×2 H_2O (g l^{-1})	3.00	na	na
NaCl (g l^{-1})	20.0	20.0	20.0
$(NH_4)_2SO_4$ (g l^{-1})	2.50	4.60	105
$MgSO_4$ (g l^{-1})	0.240	0.120	2.12
$CaCl_2$ (mg l^{-1})	11.0	6.00	72.0
L-Pro (mg l^{-1})	na	40.0	700
$FeSO_4$×$7H_2O$ (mg l^{-1})	4.87	3.60	63.9
$MnCl_2$×$4H_2O$ (mg l^{-1})	1.50	2.25	39.4
$ZnSO_4$ (mg l^{-1})	1.05	0.780	13.6
H_3BO_3 (mg l^{-1})	0.300	0.300	0.300
Na_2MoO_4×$2H_2O$ (mg l^{-1})	0.250	0.250	0.250
$CuCl_2$*$2H_2O$ (mg l^{-1})	0.150	0.150	0.150
Niacinamide (mg l^{-1})	35.0	6.00	105
Thiamine hydrochloride (mg l^{-1})	8.00	1.50	26.2
Calcium pantothenate (mg l^{-1})	15.0	6.75	118
Biotin (mg l^{-1})	0.100	0.06	1.05
Riboflavine (mg l^{-1})	1.00	na	na
Kanamycin (mg l^{-1})	20.0	na	na
MOPS (mM)	na	10.0	10.0
Na_2EDTA×$2H_2O$ (µg l^{-1})	0.84	0.840	0.920
Industrol 204 (mg l^{-1})	na	0.500	20.0
Hydrochloric acid (mM)	1.00	1.00	1.00
Sulfuric acid (mM)	na	3.00	3.00

NA not added

Medium optimization in continuous culture

We used continuous culture experiments and a modified pulse and medium-shift technique (Kuhn et al. 1979; Goldberg and Er-el 1981) to obtain a medium composition that is balanced with respect to the single medium components. As an initial condition, medium sGM1 was modified as follows: per liter, 0.5 ml of antifoam and 3 mmol of H_2SO_4 were added to prevent foam formation and precipitation of medium components; Na_2HPO_4×$2H_2O$ was omitted from the medium, and L-Cys, L-Leu, L-Met, L-Pro were added. The concentrations of the other components were changed according to the experimental program (see Table 2). The continuous cultures were carried out in a 2-l stirred-tank bioreactor BiostatA equipped as described elsewhere (Valsesia et al. 2007). Cultivations were started by diluting cultures 50-fold into 50 ml of modified sGM1 medium in shake flasks and incubation at 190 rpm and 37°C for 12 h. The continuous culture was inoculated by diluting this preculture 15-fold into 0.7 L of modified medium sGM1. The temperature was set to 37°C. The pH was maintained at 6.5 by the controlled addition of 1 M aqueous NaOH. The working volume was maintained at 0.7 l by a weight-controlled peristaltic pump that removed liquid phase form the reactor when the weight crossed a preset value. A constant airflow of 2 l min^{-1} was maintained by a mass flow controller, and the stirring rate was set to values between 900 and 1,000 rpm, ensuring dissolved oxygen concentrations (DOT) above 20% at all times. After a startup batch phase of approximately 15 h, the cultivations were switched to continuous mode at a dilution rate D of 0.25 h^{-1}. This value corresponds to 70% of μ_{max} in medium sGM1, which had been determined by washout (Sonnleitner et al. 1984; data not shown). The yield coefficients were calculated according to Eq. 1 (Herbert et al. 1956):

$$Y_{X/S_i} = \left(S_{0,i} - \overline{S_i}\right)/\overline{X} \tag{1}$$

where Y_{X/S_i} is the yield of biomass on substrate i (in gram per gram), $S_{0,i}$ is the concentration of i in the feed stream, $\overline{S_i}$ is the concentration of i in the outlet stream, and $\overline{X}$ is the dry biomass concentration in the outlet stream. Since generally $\overline{S_i} << S_{0,i}$ up to high values of D, the influence of the substrate remaining in the outflow on the yield is practically negligible (Herbert et al. 1956). This leads to the simplified equation (Mateles and Battat 1974):

$$Y_{X/S_i} = \overline{X}/S_{0,i}. \tag{2}$$

To implement a specific nutrient limitation, the concentration $S_{0,i}$ in the feed medium was changed until $\overline{S_i}$ became limiting and a new steady-state biomass concentration was established. For the calculation of Y_{X/S_i}, $\overline{X}$ was measured after at least five volume changes after the change in substrate concentration to ensure steady-state conditions. In order to confirm that the substrate in question was indeed limiting, a concentrated solution of the supposed substrate was pulsed into the reactor after biomass determination. The substrate was considered limiting if the pulse caused an increase of $\overline{X}$.

Fed-batch cultivations

Fed-batch fermentations were carried out in defined medium in a 10-l bioreactor BiostatA (Sartorius BBI Systems, Melsungen, Germany), again equipped as described before (Medaglia et al. 2010) The reactor, containing initially 3 l of medium sGM2 (Table 1), was inoculated with 50 ml of preculture as described above. The pH was maintained at 6.5 by the addition of 3 M NaOH or 3 M H_2SO_4. An exponential feeding was started

Table 2 Nutrient concentrations in the determination of yield coefficients

Compound	Limiting element or ion	$S_{i,\mathrm{low}}$ (mgl^{-1})	$\overline{X}$ (gl^{-1})	$Y_{X/S,1}$ (gg^{-1})	$Y_{X/S,2}$ (gg^{-1})
Maltose	C	$22.5 \cdot 10^3$	10.3	0.46	0.46
$(NH_4)_2SO_4$	N	$5.00 \cdot 10^3$	9.01	1.80	1.80
KH_2PO_4	P	$1.00 \cdot 10^3$	6.82	6.82	6.82
Niacinamide		3.50	9.70	$2.77 \cdot 10^3$	$2.00 \cdot 10^3$
Biotin		0.010	7.59	$7.59 \cdot 10^5$	$2.50 \cdot 10^5$
Thiamine hydrochloride		0.800	9.89	$1.40 \cdot 10^4$	$1.00 \cdot 10^4$
Calcium pantothenate		1.50	8.14	$5.90 \cdot 10^3$	$2.22 \cdot 10^3$
$MgSO_4 \times 7\ H_2O$	Mg^{2+}	24.0	3.64	$1.52 \cdot 10^2$	$1.25 \cdot 10^2$
$FeSO_4$ x $7H_2O$	Fe^{2+}	0.480	4.20	$8.75 \cdot 10^3$	$4.16 \cdot 10^3$
$ZnSO_4 \times H_2O$	Zn^{2+}	0.200	8.19	$4.09 \cdot 10^4$	$2.5 \cdot 10^4$
$MnCl_2 \times 4H_2O$	Mn^{2+}	0.375	6.28	$1.67 \cdot 10^4$	$6.66 \cdot 10^3$
$CaCl_2$	Ca^{2+}	0.040	10.3	$>2.58 \cdot 10^5$	$>2.58 \cdot 10^5$

$S_{i,low}$ substrate concentration necessary to obtain a limitation for substrate i, $\overline{X}$ steady-state biomass concentration obtained at substrate concentration $S_{i,\mathrm{low}}$, $Y_{X/S,1}$ biomass yield per unit of substrate consumed obtained at substrate concentration $S_{i,\mathrm{low}}$; $Y_{X/S,2}$ biomass yield per unit of substrate consumed calculated once the chemostat was again phosphorus limited (see text)

17 h after inoculum, shortly before maltose depletion. The feeding rate followed Eq. 3 (Lee 1996):

$$\mathrm{Feed}(t) = \left(\frac{\mu_{\mathrm{set}}}{Y_{X/S}}\right) X_0 \frac{V_0}{S_0} e^{\mu_{\mathrm{set}} t} \qquad (3)$$

where Feed (in liter per hour) is the feeding rate, t is the time elapsed from beginning of the exponential feed, μ_{set} (h^{-1}) is the desired specific growth rate, $Y_{X/S}$ (g g^{-1}) is the biomass yield per unit of maltose consumed (we used the value of 0.46 g g^{-1} for the yield of CDW on maltose, as obtained in the medium development experiments, see below), X_0 (g l^{-1}) is the CDW concentration at the beginning of the feed, V_0 (l) is the liquid volume in the reactor at the start of the feed, S_0 (g l^{-1}) is the concentration of the limiting substrate maltose in the feeding solution fGM2 (Table 1), and t (h) is the time that has elapsed since the start of the feed. To ensure a DOT above 20%, first the stirrer speed and then the aeration rate were manually progressively increased to maximum values of 1,200 rpm and two volumes per volume per minute, respectively.

Off-line analytical procedures

Maltose, acetate, GDM, and PGDM were quantified as described elsewhere (Medaglia et al. 2010). For structural characterization of PGDM and the products of the tryptic digest, samples were analyzed after product isolation by MALDI-TOF on an Autoflex II machine (Bruker, Bremen, Germany) using a matrix composed of sinapinic acid saturated in acetonitrile/0.1% aqueous trifluoroacetic acid (30/70). The calibration of the MS predicted an accuracy of 400 ppm in the mass range between 1 and 4 kDa.

Product isolation

PGDM was isolated with a protocol adapted from Allgaier et al. (1991). First, PGDM was adsorbed from the cell-free supernatant onto 8% (*w*/*v*) XAD-1180. The adsorption experiment was carried out in a stirred vessel overnight at pH 6.5, 100 rpm, and room temperature (RT). Then, the resin was filtered off, washed with 10 mM sodium phosphate (pH 6.5), and PGDM was eluted with a buffer composed of methanol/1.25 mM aqueous H_2SO_4 (60/40). After methanol evaporation in vacuo, the pH of the eluate was adjusted at 4.5 with 1 M NaOH before loading onto the strong cation exchanger SP-sepharose Fast Flow (GE Healthcare, Uppsala, Sweden). After loading, the gel was washed with 0.1 M NaCl and eluted with a buffer composed of MeOH and an aqueous solution containing 1 M NaCl and 0.01 M HCl (30/70). Methanol was evaporated, and the solution was desalted on XAD-1180 by repeating the same procedure described above, based on adsorption on the resin, desorption with methanol/1.25 mM aqueous H_2SO_4 (60/40), and methanol evaporation in vacuo. Finally, after methanol evaporation, the eluate was lyophilized and the residue stored at 4°C.

Tryptic digest of PGDM

PGDM was digested with γ-irradiated porcine trypsin (1,000–1,500 U/mg of solid substance, based on α-*N*-benzoyl-L-arginine hydrolysis (Bergmeyer 1983) as specified by the vendor). The reaction was carried out in a 0.05 M Na-phosphate buffer at pH 6 and RT with an enzyme concentration of 100 mg l^{-1} until the main PGDM peak was no longer detectable by at-line high-performance liquid chromatography (HPLC). The conversion yield for this procedure (in the absence of other PGDM peaks, see "Results" section) is typically 95% on an analytical or on the small preparative scale required for the reported experiments. The details of the purification procedure and the enzymatic conversion will be published elsewhere.

Results

Nutritional requirements of *S. gallinarum* ΔP

Several defined media had been previously tested for the production of mature GDM by wild-type *S. gallinarum*. GDM production was supported in a medium containing several amino acids, vitamins, trace elements, and BSA with a maximum GDM titer of approximately 0.14 mM (Ungermann 1992). The addition of BSA was required to obtain detectable quantities of GDM, making the closing of amino acid balances difficult and the medium expensive. However, to our knowledge, no systematic auxotrophy study has been conducted for *S. gallinarum* to date. Starting from the medium described previously (Ungermann 1992), a literature survey regarding mineral media for staphylococci suggested a number of possibilities for further improvement: iron is required as an essential constituent in staphylococci (Marcelis et al. 1978; Lindsay and Riley 1994; Beasley et al. 2009). It is typically added to *S. aureus*-defined media (Gould and Lennarz 1970; Wu and Bergdoll 1971; Miller and Fung 1973), and it has been reported to be beneficial for the production of mature GDM by wild-type (wt) *S. gallinarum* (Hörner et al. 1990). We also limited the number of added vitamins to those five (niacinamide, thiamine, pantothenic acid, biotin and riboflavin) which are typically added in *S. aureus*-defined media (Miller and Fung 1973; Emmett and Kloos 1975; Onoue and Mori 1997). While we could not identify a systematic amino acid auxotrophy for this strain, some amino acids are known to stimulate the production of mature GDM by wt *S. gallinarum*, particularly L-Cys and L-Pro (Ungermann 1992) and L-Glu, Gly, L-Ser, and L-Thr (Kempf et al. 1999a). According to these considerations, we formulated the basal medium sGM1, containing (1) maltose as the principal organic carbon source, (2) a set of salts such as NH_4^+, Mg^{2+}, Na^+, Ca^{2+}, K^+, Mg^{2+}, NH_4^+, PO_4^{2-}, SO_4^{2-} (Madigan et al. 2000), (3) the five vitamins mentioned above, and (4) a set of trace elements based on the well-defined requirements of *Escherichia coli* (Panke et al. 1999). Initially, we added all 20 proteinogenic amino acids into the medium and *S. gallinarum* ΔP grew in shake flasks with a μ_{max} of 0.5 h l^{-1} to a CDW of 5.25 g l^{-1} while producing PGDM to 18 mg l^{-1}.

The influence of amino acids in the medium

Preliminary experiments had shown that *S. gallinarum* ΔP did not exhibit any amino acid auxotrophy; though without amino acids in the medium, it did exhibit a prolonged lag phase of approximately 10 h. Once growth started, μ_{max} reached 0.4 h^{-1}, which was only 20% lower than the value obtained in presence of the 20 proteinogenic amino acids. Remarkably, the maximum PGDM titer was not markedly influenced by the presence of the amino acids in the medium (data not shown), indicating that amino acids might not be necessary for efficient production in this medium.

To identify the most important amino acids that would be required to shorten the lag phase, we organized the 20 proteinogenic amino acids in groups according to anabolic origin and then omitted one group per experiment, and in those cases where an effect was observed, the experiment was repeated with only one amino acid missing. Clearly, proline (glutamate family) had a strongly stimulating effect on *S. gallinarum* ΔP growth (data not shown). Less stimulation was observed for leucine, methionine, and cysteine, belonging to the alanine, aspartate, and serine families, respectively. No effect was observed for the omission of the remaining 16 amino acids.

Vitamins and trace elements

Previous experiments had indicated that *S. gallinarum* ΔP did not grow in a vitamin- or trace-element-free derivative of medium sGM1 (data not shown). To determine those vitamins and metal ions that were essential for the production of PGDM, cultures were started where single compounds were omitted from medium sGM1. For these experiments, medium sGM1 was supplemented with the amino acids L-Cys, L-Leu, L-Met, and L-Pro that had been identified as the most important in lag-phase shortening as described above.

As shown in Table 3, specifically, the omission of thiamine, niacinamide, biotin, and zinc and manganese ions from medium sGM1 led to a drastic decrease in maximum cell density and specific growth rate. Also, the PGDM concentration remained under the detection limit of 5 mg l^{-1} in cultivations without these nutrients. The omission of pantothenic acid led to a maximum acetate accumulation of 1.83 g l^{-1}, while acetate accumulation had remained below the detection limit of 0.5 g l^{-1} for all the other cultures. This resulted in lower maximum biomass concentration (X_{max}; Table 3). The omission of iron reduced X_{max} by 70% relative to the positive control, and PGDM could no longer be detected in the culture. Therefore, niacinamide, thiamine, pantothenate, biotin, iron, manganese, and zinc appeared to be essential compounds for efficient growth of *S. gallinarum* ΔP and production of PGDM.

Medium optimization in chemostat

After fixing the ingredients for a defined medium for *S. gallinarum* ΔP that still allowed efficient PGDM production, we investigated the requirements for a medium in which the amounts of the various nutrients would be

Table 3 Effect of the addition of vitamins and trace elements to medium sGM1 on growth of *S. gallinarum ΔP* and PGDM production in shake flasks.

Cultivation	X_{mas} (h^{-1})	μ_{max} (gl^{-1})	$PGDM_{max}$ (μM)	$Y_{PFDM/X,rel}$ (g/g%)	Ac_{max} (gl^{-1})	$Y_{Ac/X}$ (gg^{-1})
Positive control w/ all 5 vitamins	5.21±0.04	0.31±0.02	26±1	100	<0.5	<0.10
(w/o) niacinamide	0.06±0.00	0.02±0.01	<3	<11	<0.5	<0.10
(w/o) thiamine	0.09±0.01	0.02±0.01	<3	<11	<0.5	<0.10
(w/o) pantothenic acid	2.94±0.11	0.26±0.03	<3	25	1.83±0.05	0.31±0.04
(w/o) biotin	0.19±0.09	0.16±0.01	<3	<11	<0.5	<0.10
(w/o) riboflavin	5.48±0.16	0.25±0.02	24±2	100	<0.5	<0.10
Negative control w/o vitamins	0.02±0.00	0.01±0.00	<3	<11	<0.5	<0.10
All metals	4.85±0.07	0.24±0.02	17.4±1	100	<0.5	<0.10
(w/o) iron	3.38±0.08	0.18±0.02	<3	<17	<0.5	<0.10
(w/o) copper	4.53±0.24	0.20±0.03	18.9±1	100	<0.5	<0.10
(w/o) boron	5.05±0.08	0.21±0.01	21.6±1	100	<0.5	<0.10
(w/o) zinc	2.84±0.23	0.14±0.02	<3	<17	<0.5	<0.10
(w/o) molybdenum	4.95±0.29	0.21±0.02	17.1±2	100	<0.5	<0.10
(w/o) manganese	2.76±0.07	0.16±0.03	<3	<17	<0.5	<0.10
Negative control w/o metals	0.06±0.11	0.02±0.01	<3	<17	<0.5	<0.10

Positive controls contained all metal ions or all vitamins to be investigated. In negative controls, either all metal ions or all vitamins were omitted. The detection limits were 10 mg l^{-1} for PGDM and 0.5 g l^{-1} for acetate

w/ with, *w/o* without, X_{max} maximum biomass concentration, μ_{max} maximum specific growth rate, $PGDM_{max}$ maximum PGDM concentration measured in the same samples from which X_{max} was determined, $Y_{PGDM/X}$ PGDM yield per unit of biomass relative to the positive control for which the absolute value was 0.017 (g g^{-1}), Ac_{max} maximum acetate concentration measured in the same samples as X_{max}, $Y_{Ac/maltose}$ acetate yield per maltose consumed

balanced relative to the amount of carbon source. The required Y_{X/S_i} values for the important nutrients could be obtained from a series of chemostats. First, we determined Y_{X/S_i} for maltose, ammonium sulfate, and potassium dihydrogen phosphate. The initial concentrations for these components were set to 22.5 g l^{-1} (maltose), 5 g l^{-1} (ammonium sulfate), and 1 g l^{-1} (potassium dihydrogen phosphate) and subsequently varied in order to provoke a new limitation. Once a new steady-state biomass concentration was obtained, the nature of the limiting substrate was identified by pulse technique, and its Y_{X/S_i} could be calculated. For the other compounds, the concentrations remained as defined for medium sGM1 (Table 1). Again, the most important amino acids for growth L-Cys, L-Leu, L-Met, and L-Pro were added into medium. We found an elemental mass ratio of C/N/P of 1.000:0.128:0.036 g.

In the following, we determined the $Y_{X/S}$ for vitamins and trace metals in a series of chemostats with a modified pulse and medium-shift technique (Kuhn et al. 1979; Goldberg and Er-el 1981). For each compound, the investigation was started under P limitation (allowing for a cell density of 10 g l^{-1}), and the concentration of the nutrient under study was reduced repeatedly by tenfold until the biomass concentration decreased, indicating that this nutrient became the new limiting substrate, and a first yield coefficient ($Y_{X/S,1}$) was calculated. This coefficient allowed calculating the substrate concentration that would be required to return to the reference biomass concentration of 10 g l^{-1}. This substrate concentration was then increased by 50% to prevent multiple limitations later.

However, in a number of cases, even this increased substrate concentration was not enough to re-establish a biomass concentration of 10 g l^{-1}, indicating that the yield coefficient changed with the availability of the substrate. In these cases, we introduced a second yield coefficient ($Y_{X/S,2}$) calculated with the experimental substrate concentration necessary to re-establish a biomass concentration of 10 g l^{-1}. The highest variation was observed for biotin, where the second yield coefficient was threefold lower than the first (Table 2). Such variations can originate from contaminations introduced with other medium components or physiological changes in membrane transport as a function of external nutrient concentration.

We could not implement calcium-limited growth even though its concentration in the incoming medium was stepwise decreased to 0.04 mg l^{-1} (0.11 mg l^{-1} $CaCl_2$), equivalent to a calcium content in the biomass of less than 0.001 mg g^{-1}. In contrast, three different *Bacillus* strains (another low GC Gram-positive bacterium) have calcium contents between 0.16 and 0.39 mg g^{-1} (Mateles and Battat 1974; Kuhn et al. 1979; Summers et al. 1979). Again, we contribute this effect to Ca^{2+} impurities of other medium components. As a precautionary measure, we assumed a yield coefficient for $CaCl_2$ of only 500 g g^{-1} in subsequent media formulations.

The investigation also confirmed the strong influence of specific vitamins on acetate accumulation. Limiting the pantothenic acid or biotin concentration led to an increase of the acetate yield per maltose consumed ($Y_{Ac/maltose}$), which increased by factors of 4 and 2, respectively (Fig. 1).

Fed-batch cultivations with *S. gallinarum* ΔP

The balanced defined medium developed above was next used to establish a fed-batch cultivation. The batch-phase medium sGM2 (Table 1) was based on the essential micronutrient contents determined by continuous culture and was designed for a phosphorus limitation. This approach was followed in order to avoid carbon limitation during the overnight culture, which could have led to product degradation as we had observed in complex media previously (Medaglia et al. 2010). In the feed medium fGM2, the required components were concentrated to allow final biomass concentrations of approximately 80 g l^{-1}. The feed medium was designed for a maltose limitation in order to enable growth control via the feed and to reduce acetate formation during feed operation (Table 1). As expected, the initial batch phase in defined medium took longer than in complex medium, 16 h instead of 4 h. The exponential feeding was started after 16.2 h. At that point, maltose was not completely depleted.

Although maximum specific growth rates up to 0.325 h^{-1} had been measured in previous continuous cultures, the feed rate of the exponential feeding μ_{set} was set to 0.15 h^{-1} in order to prevent overflow metabolism. Acetic acid formation was detected only between 15 and 22 h, when the remaining maltose from the batch-phase delayed the onset of the carbon source limitation. After this period, acetic acid was assimilated. This period was accompanied by a transient stop in PGDM production, indicating a not-yet-understood link of acetate metabolism to PGDM production (Fig. 2). After acetate assimilation, the cells grew with carbon-limited feeding until the end of the cultivation, and no acetate accumulation was detected anymore. In addition, we did not detect any lactate production.

Biomass concentration and PGDM accumulated up to 60 g l^{-1} and 0.6 mM 40 h after inoculation. Then, foam

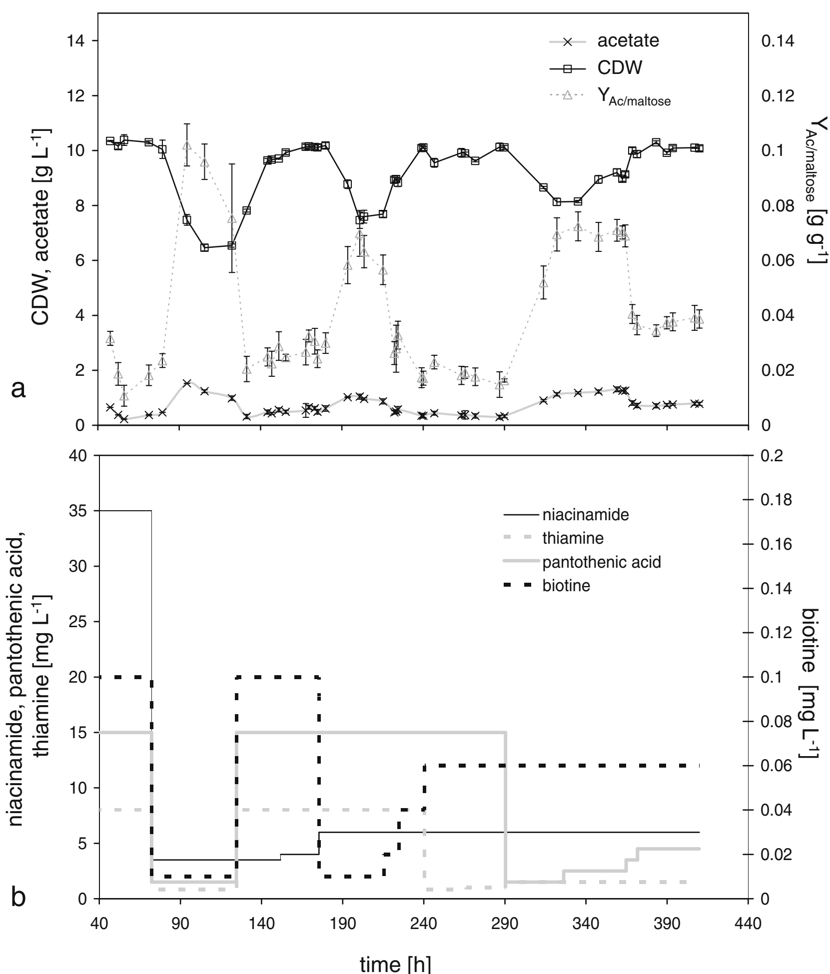

Fig. 1 Medium optimization for vitamins. **a** Biomass concentration, acetate concentration, and acetate yield per maltose consumed ($Y_{Ac/maltose}$) as a function of vitamin concentration. **b** Vitamin concentrations in the feed medium

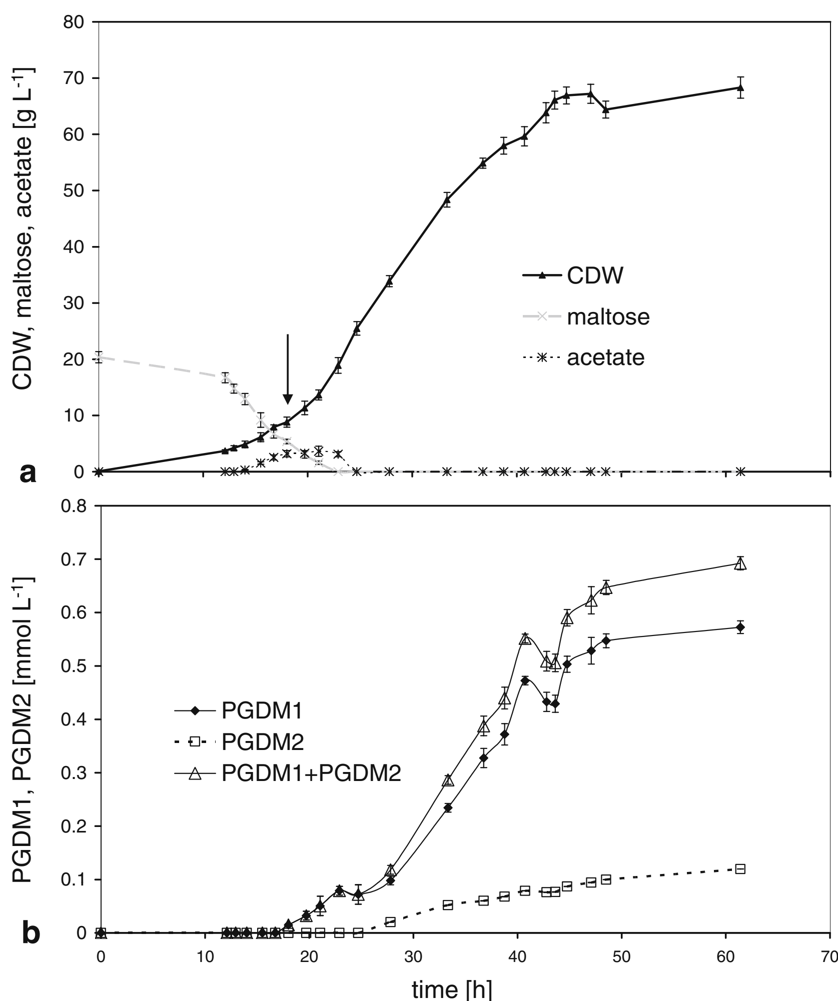

Fig. 2 *S. gallinarum ΔP* fed-batch fermentation in defined medium. Time courses of **a** biomass, maltose, and acetate concentrations and **b** PGDM1, PGDM2, and total PGDM (PGDM1 + PGDM2) concentrations. The maximum PGDM concentration of 0.69 mM corresponds to a GDM concentration of 1.49 g l^{-1}. The *arrow* indicates the start of the feeding

formation required a reduction of the feed rate to ultimately 0.46 g l^{-1} after 48 h, which no longer sustained increases in biomass and PGDM concentrations, and the cultivation was terminated. The maximum CDW concentration was 67 g l^{-1}. PGDM accumulated to 0.57 mM (1.95 g l^{-1}). As described below, a novel pregallidermin peak appeared in the HPLC analysis. Including this in the calculation, the total precursor concentration was 0.69 mM.

Fed-batch cultivations with wild-type *S. gallinarum*

To obtain a comparable data set to assess the potential advantage of PGDM production over the direct production of GDM, we compared both under similar production conditions and applied the established fed-batch protocol to the wild-type strain *S. gallinarum* and GDM production. During batch phase, the wild-type strain grew faster than the mutant strain, and no acetate was produced. Therefore, maltose feed was started earlier, after 13.8 h before its depletion in the batch medium. Unexpectedly, acetate accumulation was detected after 21 h, and the feed rate was switched from exponential to linear regime and maintained at 3.5 $g_{maltose}$ l^{-1} h^{-1} between 20 and 38 h, when feeding was stopped because GDM was no longer produced. Unlike in previous fermentations with *S. gallinarum* in complex media, both PGDM and GDM were detected in the medium (Fig. 3). The maximum GDM concentration reached in this cultivation was 0.070 mM (170 mg l^{-1}). After correction for volume increase through feeding, it becomes clear that the sum of PGDM and GDM amounts remained constant, with PGDM decreasing and GDM increasing (Fig. 3b). This suggests that the biosynthesis of (P)GDM leveled off 20 h after inoculation, and the changes in absolute amounts were only due to conversion of PGDM to GDM.

PGDM characterization

As described above, at high overall PGDM concentrations during fed-batch cultivations of *S. gallinarum ΔP*, we detected an additional unknown product peak with a slightly higher retention factor (R_f=8.63) than the PGDM we had observed previously (referred to as PGDM 1 below,

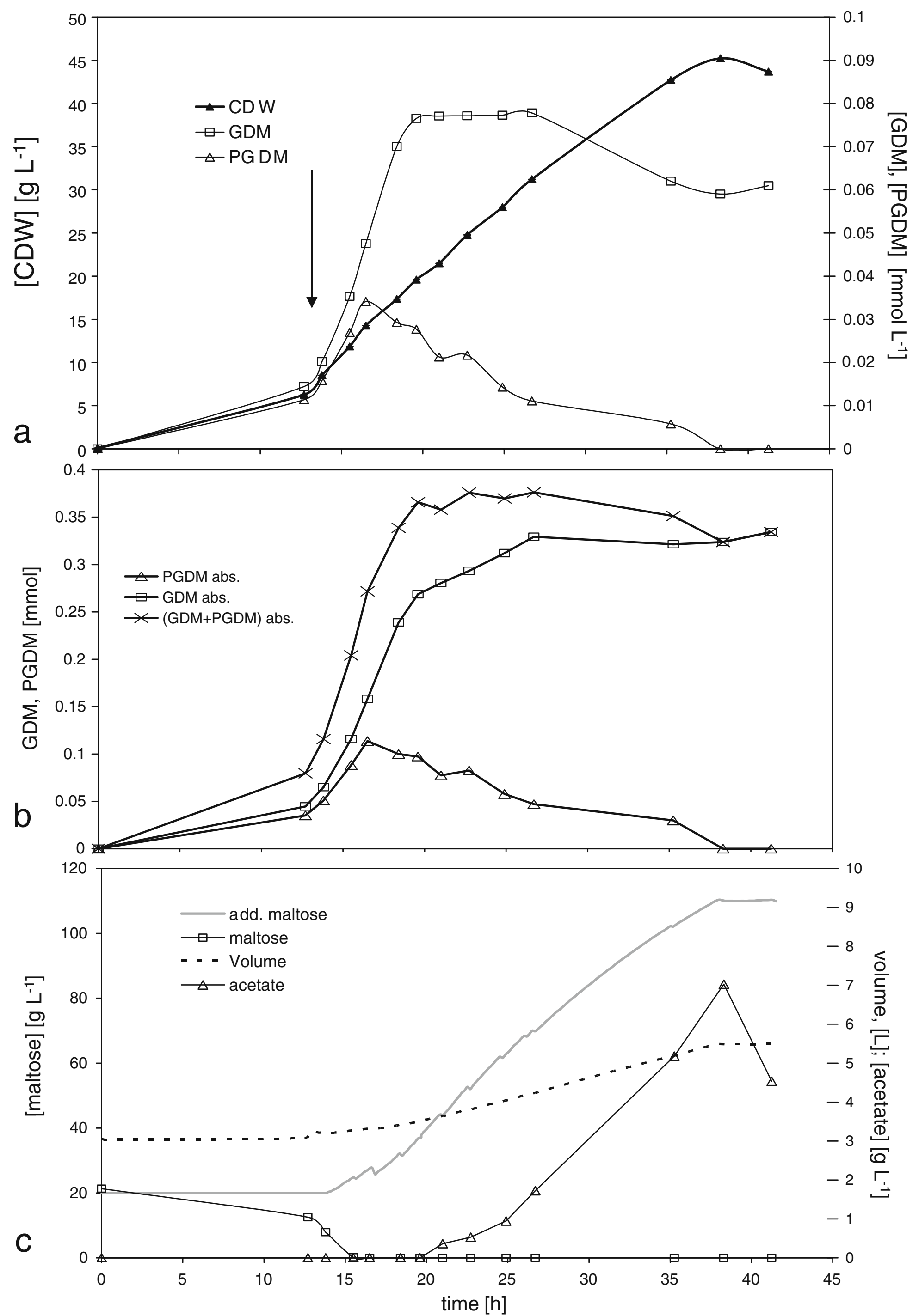

Fig. 3 Fed-batch fermentation of wild-type *S. gallinarum* in defined medium. **a** Time course of GDM, PGDM, and biomass concentrations. **b** Absolute amounts of PGDM and GDM in the reactor and relative sum. **c** Maltose addition and volume profiles; maltose and acetate concentrations in the medium. The *arrow* indicates the time where feeding was started

R_f=8.29). We observed that this product, termed PGDM 2, could be also digested by trypsin (Fig. 4), suggesting that it might also be a GDM precursor.

To confirm that PGDM2 represented indeed GDM precursors, both PGDM1 and PGDM2 were isolated together from a cell-free supernatant and then analyzed by mass spectrometry before and after tryptic digest. In samples from before the tryptic digest, we could identify most prominently the expected mass ion peaks for GDM with 12- and 14-amino-acid leader sequences ($PGDM_{12}$ and $PGDM_{14}$; Valsesia et al. 2007). Furthermore, we identified several additional *m*/*z* signals that corresponded to gallidermin with different N-terminal extensions ($PGDM_6$, $PGDM_7$, $PGDM_9$, and $PGDM_{11}$). Though the inherent imprecision on our MS measurement (400 ppm) did not allow us to approximate the masses by more than one mass unit, the agreement of *m*/*z* signals and the expected masses from subsequent N-terminal amino acid removals were striking.

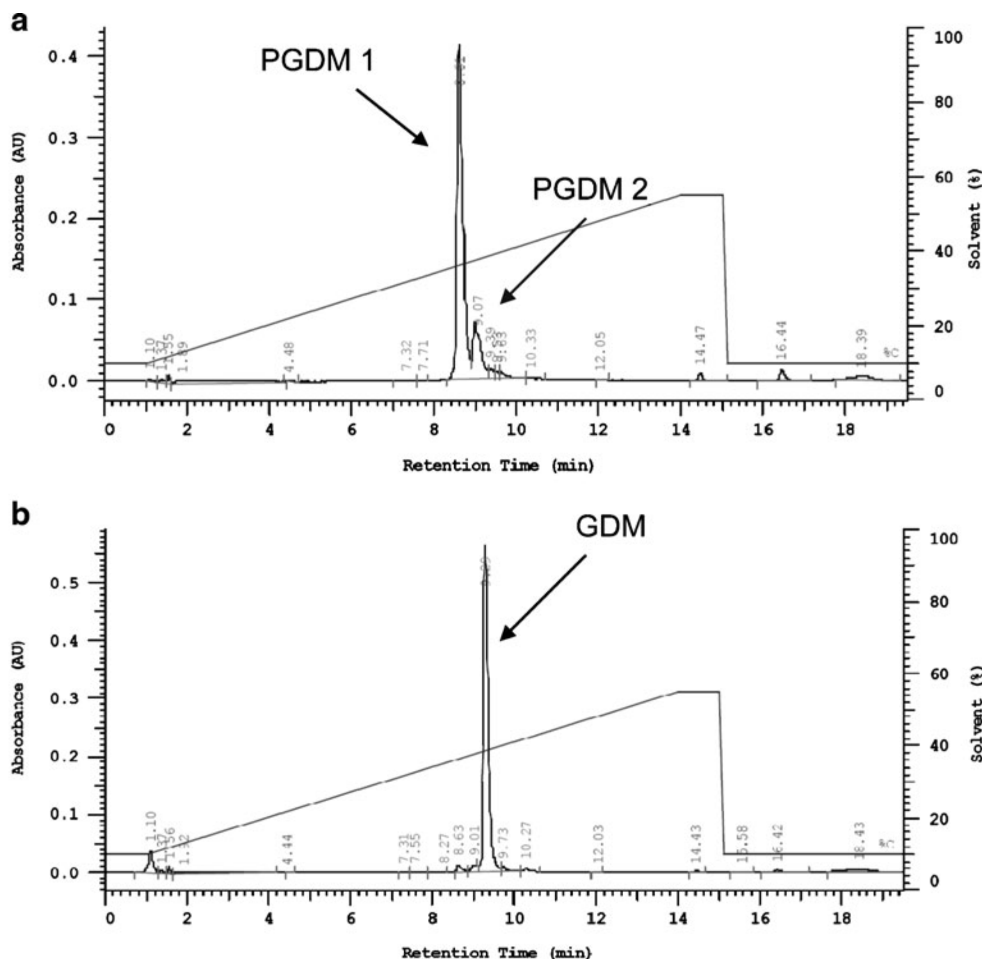

Fig. 4 HPLC chromatograms of product preparations before **a** and after **b** tryptic digest (UV-VIS detection at λ= 230 nm). **a** PGDM1 and PGDM2 before tryptic digest (UV-VIS detection at λ= 230 nm). **b** GDM after tryptic digest (λ=230 nm). Profiles: acetonitrile gradient in the mobile phase; *AU* adsorption units

After the tryptic digest, only the *m*/*z* signal for mature GDM remained. From HPLC analysis, it resulted that the amount of GDM detected was by 10% larger than could be expected from the conversion of PGDM1 alone. This suggested—though did not prove conclusively—that the various peaks had been converted by trypsin to GDM. Therefore, we felt justified to consider the additional product signals as PGDM variants that would add to the total amount of PGDMs produced during the cultivation.

For quantification of the additional amount of PGDM represented by PGDM2, we correlated the PGDM2 peak with the additional GDM that had been found in the HPLC analysis under the assumption of essentially quantitative conversion by trypsin. We found this assumption generally justified in our trypsination experiments (manuscript in preparation), and in any case it is a conservative assumption, potentially underestimating the amount of PGDM produced.

Inhibition of *S. gallinarum* ΔP by PGDM variants

The production strategy for GDM that underlies the experiments presented here relies on the production of nontoxic PGDM, which is subsequently, in a separate step, cleaved off to yield active GDM. Therefore, it was important to investigate whether the appearance of PGDM variants with an even shorter leader peptide than the already-tested 12 to 14 amino acids had an influence on the toxicity profile of the accumulating product. For this, we cultivated *S. gallinarum* ΔP in liquid cultures in medium sGM1 with 40 mg l^{-1} L-Pro and added the same PGDM mixture analyzed above. No growth inhibitory effects could be observed up to a concentration of 1.2 mM (4 g l^{-1}) PGDM1 (and correspondingly more of total PGDM variants). In contrast, the growth of *S. gallinarum* ΔP under the same conditions was already inhibited by the addition of approximately 10 mg l^{-1} of GDM obtained from the PGDM preparation after tryptic digest (data not shown). We conclude that the PGDM mixture produced in our fermentations was not toxic to the producer in the range of concentrations tested.

Discussion

Defined media are important in process development because (1) they are the basis of a rational medium

development based on detailed knowledge of strain physiology and accurate process analytics; (2) they prevent the unintended accumulation of unconsumed nutrients to inhibitory levels in fed-batch protocols; (3) they prevent intractable interference of superfluous medium components with gene regulation that might be detrimental to process productivity; (4) they allow feed-based growth control; (5) they make the process independent from variations of the composition of the complex components; and (6) they simplify product purification (Yee and Blanch 1993; Zhang and Greasham 1999; Nowruzi et al. 2008). The latter point might be fundamental for the economy of the process: generally speaking, the purification of peptides is very expensive, and there is a lack of efficient purification processes (Hongjian et al. 2006).

Systematic investigation of the amino acid requirements showed that *S. gallinarum ΔP* did not exhibit any amino acid auxotrophy in the presence of seven trace metals and four vitamins, which is in contrast with the several auxotrophies that have been observed in other staphylococci. However, auxotrophies in *S. aureus* are commonly not due to the absence of genetic information for the corresponding pathways but rather due to their repression. As a result, auxotrophies can be eliminated by successively reducing the number of amino acids in the medium (Gladstone 1937), which could explain the long lag phases observed in this study, where *S. gallinarum ΔP* had to adapt to amino-acid-free media. In fact, in *S. aureus* N135, all the genes coding for the 20 proteinogenic amino acids pathways have been annotated in the sequenced genome (Kuroda et al. 2001). However, previous studies on staphylococcal amino acid requirements, which were conducted on 823 strains isolated from the skin of 40 different individuals, showed that amino acid additions were required, with proline, arginine, and valine as the most frequently required amino acids, followed by cysteine, leucine, and methionine (Emmett and Kloos 1975). This is in good agreement with our observations: except for arginine and valine, we found that exactly the same amino acids (particularly proline) stimulated *S. gallinarum ΔP* growth, leading to a shortened lag phase.

Regarding the vitamins, the strong influence of pantothenic acid on acetate formation can be easily explained: this vitamin is a precursor of coenzyme A, an essential cofactor which is estimated to be involved in 4% to 9% of all known cellular enzymatic activities (Begley et al. 2001; Genschel 2004; Spry et al. 2007), including the channeling of glycolytic intermediates to the tricarboxylic acid cycle (TCA), and the operation of the TCA itself. Blocks in these reactions can easily lead—directly or indirectly—to the accumulation of glycolytic intermediates that lead to acetate formation via acetyl-phosphate or decarboxylation of pyruvate by pyruvate oxidase (Wolfe 2005), which has been identified for example in *S. aureus* (Patton et al. 2005).

Limiting biotin supply also led to increased acetate formation for *S. gallinarum ΔP*. Biotin is a required cofactor for numerous carboxylases, such as pyruvate carboxylase, which catalyzes the anaplerotic formation of oxaloacetate from pyruvate (Sonenshein et al. 2002). The absence of sufficient biotin could have led to an imbalance in glycolysis and TCA activities, potentially leading again to acetate formation.

Among the metals tested, only the omissions of zinc, manganese, and iron, which are essential elements for all (investigated) living microorganisms (Madigan et al. 2000), was essential for growth and PGDM production. Iron was lacking in all the previously described defined media that had been investigated for *S. gallinarum ΔP* (Ungermann 1992), suggesting that it was provided as contamination. It is also interesting to note that EpiB, the enzyme equivalent to gallidermin-related GdmB and responsible for the dehydration of serine during epidermin biosynthesis, acts by similar mechanism as a L-serine hydratase, which contains an iron–sulfur cluster (Kupke and Götz 1996). Furthermore, recent studies on the cyclase NisC, which is involved in the posttranslational thioether bridge formation in nisin synthesis and has GdmC as an equivalent in GDM biosynthesis, showed that this enzyme contains stoichiometric amounts of zinc (Okeley et al. 2003; Li et al. 2006). This suggests that there are also PGDM-synthesis-specific arguments for including these metals.

The novel cultivation protocol also led to the detection of novel PGDM variants consisting of GDM and various leader peptides. It is likely that their occurrence is rather a result of the medium change than of the fact that the titer has increased, as we had not detected the variants before when producing high PGDM titers in complex media (Medaglia et al. 2010). Given the importance of the leader peptide in PGDM export (Schnell et al. 1988), it is likely that any detected PGDM variants were produced extracellularly. While the flexibility of the mature GDM molecule is strongly restricted by the ring structures and GDM thus relatively resistant to proteolysis, no such restriction is to be expected for the leader peptide, facilitating extracellular leader hydrolysis. In fact, Staphylococci are well known for secretion of a variety of proteases (Dubin 2002).

Clearly, the novel peptide variants did not contribute to product toxicity, leaving the basic strategy to produce a nontoxic GDM precursor instead of GDM intact. The strategy is also supported by the behavior of the wt *S. gallinarum* in the developed cultivation protocol, who accumulated GDM only to only half the level reached in optimized complex medium cultivations (Kempf et al. 2000) and one ninth of the final PGDM titer that was accumulated by *S. gallinarum ΔP*. We consider it likely

that the lower titer relative to complex medium fermentation is due to a reduced tolerance in (nearly) mineral medium. Interestingly, biosynthesis of the lantibiotic in defined medium ceased more or less exactly when acetate accumulation began. As acetate formation due to overflow metabolism seems unlikely given the absence of acetate production in batch phase, this raises the possibility that the appearance of acetate is a sign of stress provoked by accumulating GDM. In general, it seems likely that the much higher accumulation of PGDM is indeed due to the absence of product toxicity.

In terms of process performance, it is interesting to note that, in addition to the higher product titer, the specific and volumetric productivities calculated over the relevant times of feeding were also roughly twofold higher. Specific PGDM productivity in the defined medium cultivation was 0.17 μmol g^{-1} h^{-1}, and volumetric productivity was 13.6 μmol l^{-1} h^{-1} and therefore in both cases about twice as high as in complex medium fermentations (Kempf et al. 2000; Medaglia et al. 2010).

As described above, one limitation in the presented cultivation strategy was foam formation, which restricted efforts to increase the oxygen transfer rate and thus probably limited the final biomass concentration. Preliminary results, which were based on the measurement of the contact angle of PGDM aqueous solution droplets on a silicium plate, show that PGDM lowers the surface tension in aqueous solutions (data not shown), suggesting that this molecule acts as a surfactant and thus contributes to the foam generation problem. This limitation could be met by either the implementation of efficient techniques to control foam or, more directly, by increasing the oxygen content of the provided air.

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