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The utilization of casein and amino acids by *Streptococcus sanguis* P_4A_7 in continuous culture

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Streptococcus sanguis P_4A_7 was grown in glucose limited conditions in continuous culture at pH 7·0 in a chemically defined medium containing either free amino acids or casein as the organic nitrogen source. Apart from aspartate and threonine, which were poorly utilized at the higher dilution rates, all amino acids in the free-amino-acid medium were utilized to various extents. At the higher dilution rates, aspartate actually increased in concentration, probably due to deamidation of asparagine. The amino acid most utilized at all dilution rates was arginine, with up to 99% of the amino acid being consumed. Both casein and its α_{s1}-casein fraction supported growth at a level only slightly lower than that obtained with the free-amino-acid medium, provided that either cysteine or thioglycollate was present. With the exception of tyrosine, nearly all of the amino acyl residues of α_{s1}-casein were utilized to some degree. In general, the higher the concentration of each amino acid in the medium (whether free or as part of α_{s1}-casein) the higher the extent of utilization by _S. sanguis_ P_4A_7. Only 50% of the arginyl residues (0·16 mM) of α_{s1}-casein were utilized compared with 99% of free arginine (1·5 mM) under similar conditions, suggesting that only 50% of the α_{s1}-casein arginine was accessible to the organism. From a comparison of the amino acid composition of α_{s1}-casein with that of the high M_r fraction of the culture supernatant it was concluded that leucine, phenylalanine, lysine, histidine, arginine and valine were specifically released from α_{s1}-casein by the endo- and exopeptidase activity of _S. sanguis_ P_4A_7.

Introduction

Previous studies (van der Hoeven *et al.*, 1985*a*; Rogers *et al.*, 1986*b*, 1987) have drawn attention to the ecological significance of arginine utilization by prominent dental plaque streptococci such as *Streptococcus sanguis* and *Streptococcus milleri.* More recently Rogers *et al.* (1988) demonstrated that *S. sanguis* P_4A_7, which is auxotrophic for arginine, could process small arginine-containing peptides, converting the released arginine to ornithine via the arginine deiminase pathway. They also showed that this strain was able to grow under continuous culture conditions in a chemically defined medium containing arginine present solely in the form of a tripeptide possessing a C-terminal arginyl residue. In this context, tryptic digests of casein, which form the basis of a number of media supporting growth of many bacteria, including oral streptococci, would provide peptides possessing C-terminal arginyl and lysyl residues.

Much information has accumulated on the role of proteolytic (caseinolytic) enzymes in the growth and nutrition of group N streptococci since this is of concern to the dairy industry (Thomas & Pritchard, 1987). However, knowledge concerning casein dissimilation is, for a number of reasons, relevant to other organisms including the oral streptococci. For example, it appears likely that the combined action of proteases and glycosidases allows dental plaque bacteria to obtain their nutritional requirements from host macromolecules, albeit of largely unknown structure (Cowman *et al.*, 1979; Glenister *et al.*, 1988). However, the primary structure of casein, a dietary protein similar to some physiological substrates, is known (Swaisgood, 1982) and this facilitates studies on the ways in which it is dissimilated by bacteria. Of particular relevance to oral streptococci is the finding that casein, α_{s1}-casein and its tryptic peptides are incorporated into dental plaque and are degraded (Reynolds, 1987).

Another area in which bacterial dissimilation of complex substrates has relevance is that of virulence mechanisms. Smith (1988) has indicated the paucity of information on the influence of bacterial nutrition and

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metabolism on virulence and has stressed the need to study nutrients that promote or retard bacterial growth *in vivo*, and to investigate their influence on bacterial components and virulence determinants in continuous cultures.

In the present study, we have investigated the ability of a strain of *S. sanguis* to utilize α_{s1}-casein or free amino acids when growing in continuous culture.

Methods

Organism. Streptococcus sanguis P_4A_7 was obtained from J. van der Hoeven, University of Nijmegen, The Netherlands. Stock cultures were maintained at −80 °C and their purity and identity were periodically checked.

Media. Initially, a filter-sterilized, chemically defined medium (CDM) was used to grow the organism (van der Hoeven *et al.*, 1985*b*). The medium contained a number of vitamins, nucleoside bases, salts, amino acids and 10 mM-glucose to give glucose-limiting conditions. Growth media subsequently used were based upon CDM containing no amino acids except cysteine; the organic nitrogen (amino acid) source was either sodium caseinate (Murray Goulburn Ltd) or the α_{s1}-casein fraction, prepared as described previously (Reynolds & Wong, 1983; Reynolds, 1987). All additives were filter-sterilized (Durapore, Millipore). CDM amino-acid-free medium was supplemented (in g l^{-1}) as follows: (*a*) sodium caseinate (1·0), cysteine (0·2); (*b*) α_{s1}-casein (0·5), cysteine (0·2); (*c*) α_{s1}-casein (0·5), sodium thioglycollate (0·5); (*d*) α_{s1}-casein (0·5); (*e*) α_{s1}-casein (0·5), 2-mercaptoethanol (0·5); and (*f*) α_{s1}-casein (0·5), methionine (0·5) (see Fig. 1).

Growth conditions. S. sanguis P_4A_7 was grown in a chemostat with a working capacity of 38 ml. The temperature was maintained at 37 °C and the pH at 7·0 by the automatic addition of 2 M-KOH. Cultures were gassed with N_2 containing 5% (v/v) CO_2 (flow-rate, 200 ml min^{-1}). Medium flow was controlled with a peristaltic pump (Pharmacia). The dilution rate (D) was set between 0·05 and 0·69 h^{-1} and the culture was allowed to equilibrate for at least 10 generations before sampling, or changing the growth medium or dilution rate. Culture purity and enumeration after ultrasonic chain dispersal were determined by plating on mitis-salivarius (Difco) and Diagnostic Sensitivity Test (Oxoid) agar plates which were incubated at 37 °C in an N_2/CO_2 (95 : 5, v/v) atmosphere. Bacterial dry weights were determined as described previously (Rogers *et al.*, 1986*a*).

Growth in the various media. After steady-state growth had been initiated in CDM the reservoir medium was changed. Media were used in the following sequence: *a*, *b*, *c*, *d*, *c*, *e*, *f* and *c* (see above and Fig. 1). Each medium was used for 10 generations before changing. It should be noted that after growth on medium *a* at $D = 0{\cdot}2\ h^{-1}$, the dilution rate was increased to 0·6 h^{-1} and after equilibration, readjusted to 0·2 h^{-1}. An analysis of variance (Sokal & Rohlf, 1969) was used to statistically analyse growth parameters in CDM and the α_{s1}-casein medium *b*.

Determination of metabolites in culture filtrates. After equilibration in each medium, 2 ml samples were taken directly from the culture vessel and immediately filtered through 0·22 µm Durapore (Millipore) filters and the cell-free filtrates stored at −80 °C. Glucose (residual) in each sample was determined enzymically by the glucose oxidase method (Sigma). Amino acid compositions of filtrates were determined using the Picotag procedure (Millipore-Waters) in which the amino groups were derivatized with phenylisothiocyanate and the derivatives identified by reverse-phase high performance liquid chromatography (Brack & Reynolds, 1987). Filtrates were first fractionated by ultrafiltration using an Amicon filter (UMO5) with an M_r 500 exclusion limit. The ultrafiltrate (M_r <500 fraction) from the free-amino-acid medium (CDM) was analysed for amino acids without prior hydrolysis. The ultrafiltrate (fraction L) from the α_{s1}-casein-based medium was analysed for amino acids both with and without prior hydrolysis and the highest levels used. The ultraretentate (fraction H) was analysed after hydrolysis. The fractions were hydrolysed with 6 M-HCl vapour at 100 °C for 24 h (Brack & Reynolds, 1987).

Results

The utilization of free amino acids in CDM by *S. sanguis* P_4A_7 grown glucose limited in continuous culture is shown in Table 1. The utilization of each amino acid expressed as nmol (mg dry weight of cells)$^{-1}$ tended to decrease with increasing dilution rate. All amino acids were utilized to various extents, except for aspartate and threonine, which were poorly utilized at the higher dilution rates. Aspartate actually increased in concentration at the higher dilution rates, presumably due to the deamidation of asparagine, as the increase in aspartate was equivalent to the decrease in asparagine (Table 1). The amino acid most utilized at all dilution rates was clearly arginine. At the lower dilution rates more than 99% of the arginine in the original medium (1·5 mM) had been consumed. This corresponded to > 3 µmol arginine (mg dry weight of cells)$^{-1}$, which was several times that of any other amino acid consumed. Some growth parameters for the continuous culture of *S. sanguis* P_4A_7 grown in CDM are presented in Table 2. The specific glucose utilization rate q_s [mmol glucose (g dry weight of cells)$^{-1}$ h^{-1}] was calculated as described previously (Rogers *et al.*, 1986*a*). A linear regression of q_s against dilution rate (D) gave the maintenance energy $m = 0{\cdot}45$ mmol glucose (g dry weight of cells)$^{-1}$ h^{-1} and the true growth yield $Y_{\text{glucose}} = 45{\cdot}4$ (g dry weight of cells)$^{-1}$ (mol glucose)$^{-1}$ according to the equation $q_s = (D/Y_{\text{glucose}}) + m$ (Pirt, 1965).

The levels of growth of *S. sanguis* P_4A_7 attained on the various casein-based media are shown in Fig. 1. Both casein and its α_{s1}-casein fraction supported growth at a level only slightly lower than that obtained in CDM containing free amino acids, provided that either cysteine or thioglycollate was present. In their absence (medium *d*, Fig. 1), a steady-state growth level was maintained in α_{s1}-casein but it was much lower. Neither 2-mercaptoethanol (medium *e*) nor methionine (medium *f*) were effective substitutes for cysteine or thioglycollate. Glucose was detected only in the culture filtrates from growth on α_{s1}-casein and α_{s1}-casein plus methionine, media *d* and *f*, respectively.

When the growth rate was increased to $D = 0{\cdot}6\ h^{-1}$ in the casein medium *a*, the biomass did not decrease (Fig. 1, point A). No significant difference was found in the

Table 1. *Utilization of free amino acids by S. sanguis P_4A_7 grown in glucose limited conditions in continuous culture at various dilution rates*

Results are expressed as nmol (mg dry weight of cells)$^{-1}$ and are means of triplicate determinations. Numbers in parentheses are the amounts of amino acid consumed as a percentage of that originally present.

Amino acid	Dilution rate (h^{-1}): 0·05	0·08	0·20	0·29	0·43	0·69	Medium†
Asp	+242* (–)	12 (5·9)	125 (66·4)	+142* (–)	+36* (–)	+410* (–)	71
Glu	396 (39·6)	0 (0)	181 (20·1)	339 (43·4)	342 (43·4)	345 (26·9)	340
Ser	331 (99·4)	238 (75·2)	226 (75·3)	189 (75·0)	142 (54·3)	135 (31·6)	113
Gly	549 (49·0)	317 (29·8)	308 (30·5)	290 (34·2)	234 (26·6)	327 (22·7)	381
His	695 (99·6)	435 (95·5)	337 (78·0)	186 (51·4)	156 (41·5)	226 (36·7)	163
Arg	4176 (99·2)	3969 (99·3)	3762 (99·3)	3151 (99·1)	2953 (89·1)	1042 (19·2)	1451
Thr	77 (45·0)	24 (14·9)	27 (17·5)	3 (2·3)	0 (0)	0 (0)	59
Ala	789 (74·6)	315 (31·4)	313 (33·0)	281 (35·2)	20 (34·7)	413 (30·4)	359
Pro	301 (49·7)	167 (28·9)	160 (29·4)	145 (31·7)	117 (24·5)	156 (20·0)	206
Tyr	600 (84·0)	333 (49·1)	302 (47·0)	403 (74·7)	152 (27·0)	196 (21·3)	243
Val	219 (70·4)	150 (50·7)	155 (55·4)	185 (78·8)	96 (39·3)	103 (25·7)	106
Met	90 (71·1)	51 (42·3)	66 (58·1)	45 (47·2)	57 (57·2)	119 (73·3)	43
Cys	553 (68·1)	450 (58·3)	433 (59·3)	561 (91·5)	212 (33·3)	253 (24·2)	276
Ile	170 (70·7)	126 (55·3)	135 (62·4)	127 (69·7)	110 (57·9)	87 (28·0)	82
Leu	246 (70·0)	125 (53·4)	129 (57·8)	131 (70·4)	111 (57·6)	92 (29·0)	84
Phe	132 (84·2)	45 (30·3)	69 (48·9)	71 (59·9)	46 (36·9)	47 (23·5)	53
Lys	200 (87·3)	152 (69·5)	161 (77·1)	136 (73·3)	127 (70·5)	135 (44·5)	78
Hyp	336 (55·3)	197 (34·1)	187 (34·2)	151 (33·0)	152 (31·7)	175 (22·4)	207
Asn	243 (75·9)	119 (39·0)	131 (45·5)	152 (62·7)	86 (34·1)	412 (100)	109

* Values preceded by + indicate net production of amino acid.

† Concentration of amino acid (nmol ml^{-1}) in original medium.

Table 2. *Growth parameters of Streptococcus sanguis P_4A_7 grown in glucose-limited conditions in continuous culture with either amino acids or α_{s1}-casein as the organic nitrogen source*

Unless stated otherwise, values are the means of triplicate determinations.

Dilution rate (h^{-1})	Growth medium*	Biomass (mg l^{-1})	q_s†	$Y_{glucose}$†
0·05	A	340	1·5	34·0
0·08	A	358	2·1	35·8
0·20	A	406 ± 19‡	4·9 ± 0·2	40·6 ± 1·9‡
	C	362 ± 14	5·5 ± 0·2	36·2 ± 1·4‡
0·29	A	450	6·4	45·0
0·43	A	432	9·9	43·2
0·69	A	264	15·7	44·0

* The growth media were chemically defined and the organic nitrogen source consisted of either free amino acids (A) or α_{s1}-casein plus cysteine (C) (see Methods).

† The rate of glucose utilization (q_s) is expressed as mmol glucose (g dry weight of cells)$^{-1}$ h^{-1} and the growth yield ($Y_{glucose}$) in g dry weight of cells (mol glucose)$^{-1}$.

‡ Mean ± SD of five determinations.

maximum specific growth rate (0·8 h^{-1}) in the casein medium *a* and the free-amino-acid medium (CDM).

With the exception of tyrosine, nearly all of the amino acyl residues of α_{s1}-casein (medium *b*) were utilized to some degree. In general, the higher the concentration of each amino acid in the medium (whether free or as part of α_{s1}-casein) the higher the level of utilization by *S. sanguis* P_4A_7. Comparison of the utilization of α_{s1}-casein (Table 3) with that of free amino acids (Table 1) shows that only 50% of the arginyl residues were utilized from α_{s1}-casein [corresponding to 225 nmol (mg dry weight)$^{-1}$], whereas 99% of the free arginine was utilized [corresponding to 3762 nmol (mg dry weight)$^{-1}$] under similar conditions.

A comparison of the amino acid composition of the high-M_r fraction H ($M_r > 500$) of the culture filtrate with that of the original α_{s1}-casein medium enabled the determination of amino acids specifically released from α_{s1}-casein by *S. sanguis* P_4A_7 (Table 4). These amino acids were leucine, phenylalanine, lysine, histidine, arginine and valine. The mol% of phosphoserine in the high-M_r fraction was also less than in the original α_{s1}-casein medium while the mol% of serine was greater, suggesting that the phosphoserine had been dephosphorylated. The low-M_r fraction L ($M_r < 500$) of the culture filtrate contained predominantly free amino acids as there was little difference in levels before and after acid hydrolysis. The amino acyl residues of α_{s1}-casein that were the least utilized by *S. sanguis* P_4A_7, i.e. tyrosine, alanine and proline (Table 3), accumulated relative to the other amino acids in the low-M_r fraction

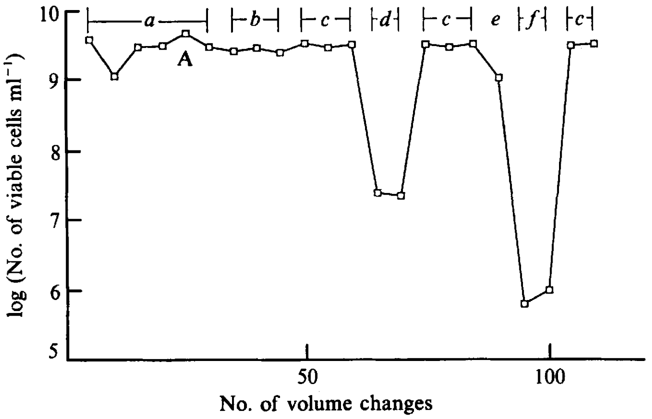

Fig. 1. Glucose-limited growth of *Streptococcus sanguis* P_4A_7 in continuous culture at dilution rate of $D = 0{\cdot}2\ h^{-1}$ with casein or α_{s1}-casein as the organic nitrogen source. Following steady-state growth in the CDM containing a range of amino acids, growth was tested in a series of CDM amino-acid-free media to which were added casein or α_{s1}-casein (see Methods). The medium reservoir was changed, after equilibration in each new medium, in the following sequence: *a*, casein plus cysteine; *b*, α_{s1}-casein plus cysteine; *c*, α_{s1}-casein plus thioglycollate; *d*, α_{s1}-casein; *e*, α_{s1}-casein plus mercaptoethanol; *f*, α_{s1}-casein plus methionine and *c*. At point A the dilution rate was increased to $D = 0{\cdot}6\ h^{-1}$ and, after equilibration, re-set at $D = 0{\cdot}2\ h^{-1}$.

Table 3. *Utilization of amino acyl residues in α_{s1}-casein by S. sanguis P_4A_7 grown under glucose-limited conditions in continuous culture at a dilution rate of $D = 0{\cdot}2\ h^{-1}$*

Residue	Concn* (nmol ml^{-1})	Utilization†
Asx	404	487 (42·8)
Thr	152	186 (43·4)
Ser	377	279 (26·3)
SerP	61	87 (50·8)
Glx	1052	839 (28·3)
Pro	456	239 (18·6)
Gly	280	214 (27·1)
Ala	287	82 (10·1)
Val	314	388 (43·9)
Met	94	104 (39·4)
Ile	295	346 (41·7)
Leu	528	574 (38·6)
Tyr	147	0 (0)
Phe	235	205 (31·1)
Lys	404	515 (45·3)
His	120	177 (52·5)
Arg	159	225 (50·3)
Cys	209	0 (0)

* The growth medium contained $0{\cdot}5\ g\ l^{-1}$ α_{s1}-casein plus added cysteine (see Methods.) Tryptophan was not analysed.

† Residue utilization is expressed as nmol (mg dry weight of cells)$^{-1}$ and (in parentheses) as a percentage of that available in α_{s1}-casein. Values are the means of triplicate determinations.

(Table 4). The other two amino acids (leucine and phenylalanine) that accumulated in fraction L were relatively well utilized by *S. sanguis* P_4A_7, therefore their accumulation is consistent with their specific release from α_{s1}-casein.

The growth parameters for the continuous culture of *S. sanguis* P_4A_7 in the α_{s1}-casein medium (*b*) are shown in Table 2. The biomass and molar growth yield $Y_{glucose}$ were slightly lower ($P < 0{\cdot}005$) than the corresponding values for growth in the free-amino-acid medium containing 1·5 mM-arginine.

Discussion

S. sanguis P_4A_7 grown in glucose-limited conditions in continuous culture utilized the free amino acids of the CDM to varying degrees, even though most are not essential for growth (Carlsson, 1972). Arginine, which is essential for growth, was almost completely utilized. This result is consistent with the previous work of Marsh *et al.* (1985), who studied *S. sanguis* NCTC 7865, and Rogers *et al.* (1986*a*, *b*), using *S. milleri* strains B448 and 699B3. The complete utilization of arginine by these organisms contrasts with the pattern of utilization of free amino acids by *Streptococcus mutans* Ingbritt (Hamilton & Ellwood, 1978) and *Actinomyces viscosus* GN431/75 (Hamilton & Ellwood, 1983), where cysteine and asparagine, respectively, were the predominant amino acids consumed. *S. sanguis* P_4A_7 has an auxotrophic requirement for arginine and also possesses the arginine deiminase pathway. Once the auxotrophic requirement is met (10 μM under these growth conditions), the excess arginine is converted to energy with the release of ornithine and ammonia (Rogers *et al.*, 1986*b*).

In the present study, we have shown that *S. sanguis* P_4A_7 can grow glucose-limited in continuous culture with casein as the sole amino acid source at both low and high dilution rates. This result is consistent with the previous demonstration that both the soluble and cell debris fractions of disrupted *S. sanguis* cells displayed caseinolytic activity (Cowman *et al.*, 1975). Various *Streptococcus cremoris* strains grow well in a casein medium at low dilution rates but at high dilution rates the rate of caseinolysis is too slow to support adequate growth and washout occurs (Hugenholtz *et al.*, 1987). In the present study, the rate of caseinolysis by *S. sanguis* P_4A_7 was sufficiently high to allow growth at μ_{max} ($0{\cdot}8\ h^{-1}$).

Other studies with *S. cremoris* HP have shown that *β*-casein but not α_{s1}-casein is degraded (Geis *et al.*, 1986; Exterkate & De Veer, 1987). In contrast, we found that *S. sanguis* P_4A_7 grew as well on α_{s1}-casein as on whole casein. However, such growth was greatly depressed in

Table 4. *Amino acid compositions of M_r fractions from a filtrate of a glucose-limited continuous culture of Streptococcus sanguis P_4A_7 grown at a dilution rate of $D = 0{\cdot}2\ h^{-1}$ in a medium containing α_{s1}-casein*

	Concn (nmol ml^{-1})		Composition (mol%)		
Residue*	Fraction L ($M_r < 500$)	Fraction H ($M_r > 500$)	Fraction L ($M_r < 500$)	Fraction H ($M_r > 500$)	α_{s1}-casein
Asx	16	215	2·31	7·37	7·71
Thr	ND	86	ND	2·95	2·91
Ser	10	268	1·44	9·18	7·20
SerP	9	21	1·30	0·72	1·17
Glx	131	623	18·90	21·35	20·10
Pro	119	252	17·17	8·64	8·71
Gly	17	187	2·45	6·41	5·34
Ala	61	197	8·80	6·75	5·47
Val	16	160	2·31	5·48	6·00
Met	3	54	0·43	1·85	1·80
Ile	3	169	0·43	5·79	5·64
Leu	137	187	19·77	6·41	7·62
Tyr	78	73	11·26	2·50	2·80
Phe	64	98	9·24	3·36	4·48
Lys	17	204	2·45	6·99	7·72
His	6	51	0·87	1·75	2·29
Arg	6	73	0·87	2·50	3·03

ND, Not detected.
* Tryptophan was not analysed.

the absence of either cysteine or thioglycollate. Other workers (Cowman *et al.*, 1975; Carlsson, 1972) have demonstrated a requirement for cysteine in various batch-grown strains of *S. sanguis*. For example, Cowman *et al.* (1975) showed that dithiothreitol could not replace cysteine. However, in the present study we found that with *S. sanguis* P_4A_7 thioglycollate could replace cysteine. This, together with the finding that cysteine added to the α_{s1}-casein medium was poorly utilized by *S. sanguis* P_4A_7 under the experimental conditions used, indicates that cysteine functions more as a reducing agent than as a nutrient. Glucose was detected only in the culture filtrates from growth on α_{s1}-casein and α_{s1}-casein plus methionine, i.e. media not containing a reducing agent. As steady-state growth was obtained with these media, albeit with a markedly reduced biomass, this suggests that growth was limited by a factor other than glucose. One possible explanation is that the role of the reducing agent is to detoxify H_2O_2 produced and that without this mechanism growth would be limited by the H_2O_2 level and therefore restricted to a small biomass.

Only 50% (0·08 mM) of the arginyl residues of α_{s1}-casein were utilized by *S. sanguis* P_4A_7, whereas more than 99% of free arginine (1·5 mM) was consumed. This result suggests that not all of the α_{s1}-casein arginine was accessible to the organism, either because the rate of release of all arginyl residues was too slow or that only half of the residues were released by the organism's endo- and exopeptidases. From a comparison of the amino acid composition of α_{s1}-casein with that of the high-M_r fraction of the culture supernatant (Table 4) it can be seen that leucine, phenylalanine, lysine, histidine, arginine and valine were specifically released from α_{s1}-casein by the organism's endo- and exopeptidase activities. This suggests that some of the cleavage sites of the α_{s1}-casein molecule (Swaisgood, 1982) are within the following sequences: residues 20–25 [Leu-Leu-Arg-Phe-Phe-Val], residues 98–103 [Leu-Leu-Arg-Leu-Lys-Lys] and residues 119–121 [Arg-Leu-His] which account for 50% of the six arginyl residues of α_{s1}-casein (Swaisgood, 1982).

The growth characteristics of *S. sanguis* P_4A_7 cultured in CDM are very similar to those found previously for strains of *S. milleri* and mutans streptococci (Rogers *et al.*, 1986*a*). The molar growth yield, Y_{glucose}, obtained with the α_{s1}-casein medium was slightly lower (11%) than that obtained with the CDM. As end-products were not measured the reason for this slightly lower molar growth yield cannot be determined; however, it is likely that it can be, in part, attributed to the lower level and availability of arginine in the α_{s1}-casein medium.

Work is currently underway to purify and characterize the endopeptidase activity of *S. sanguis* P_4A_7.

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