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Pigment Production by *Streptococcus agalactiae* in Quasi-Defined Media

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A quasi-defined medium that supports the growth of *Streptococcus agalactiae* as pigmented colonies has been developed. The medium contains starch, a peptic digest of albumin, amino acids, nucleosides, vitamins, and salts. The presence of free cysteine, which could be replaced with other sulphur-containing compounds and to a lesser degree by reducing agents, was required for pigment formation.

Streptococcus agalactiae (Group B streptococci [GBS]) is a leading cause of infections in newborns and adults (14). Production of an orange-red pigment integrated in the cell wall (5) is a specific characteristic of human hemolytic GBS and serves as the basis for use of culture media to identify GBS from clinical samples (2, 9).

Media for GBS pigment detection are complex, since they must contain starch (2, 5, 16), serum (2), a specific peptone (proteose peptone no. 3; Becton Dickinson, Difco, Franklin Lakes, N.J.) (2, 4, 16), and a folate pathway inhibitor (8). The component that accounts for the serum pigment-enhancing activity is amylase (10). The active peptide from peptones necessary for pigment production is Ile-Ala-Arg-Arg-His-Pro-Tyr-Phe (11), and this peptide is produced when albumin is hydrolyzed with pepsin.

Though some defined media for growth of streptococci have been described previously (6, 18, 20), in the few published studies of the dynamics of pigment production, GBS have been grown in complex media (15, 16, 17). The present study was designed to devise simpler media able to support growth and pigment production by GBS. This should aid improvement of media for GBS pigment detection and future investigation into the metabolic pathway that leads to pigment production.

Three beta-hemolytic GBS strains were used to develop the new media. Two of them were isolated from patients and identified by accepted procedures (Gram stain, beta-hemolysis, detection of group B antigen, and CAMP test) (13), and the other was *S. agalactiae* ATCC 12386. Experiments aimed at developing the new quasi-defined media were repeated at least three times. The ability of these media to support GBS growth and GBS pigment production was also tested using another 20 GBS strains isolated from clinical samples. Plates of the media were inoculated with 10 μl of a suspension prepared by homogenizing a colony from a blood agar culture in 10 ml of sterile 0.85% NaCl. Pigment was detected by inspection of the surfaces of plates after 24 h at 36°C in anaerobiosis or under a coverslide (12). Pigment intensity was graded from 0 (no pigment) to 4+ (strong red, as with colonies of strain ATCC 12386 after 24 h of anaerobic incubation in Granada medium).

We assayed first an agar medium (CAM medium) derived from Granada medium (9) in which proteose peptone no. 3, horse serum, colistin, crystal violet, and metronidazole were not present and the only components not completely defined were casamino acids and albumin digest. CAM medium contained, per liter, the following: 10 g of purified agar (Oxoid, Basingstoke, United Kingdom); 10 g of peptic digest of albumin prepared by hydrolyzing a 5% solution of bovine albumin (fatty acid free) (Sigma Chemical Co., St. Louis, Mo.) with hog pepsin (1:60,000; Sigma) (0.05 mg/mg of protein) at 50°C for 20 h, after adjusting the pH to 2.5 with HCl; 10 g of soluble starch (1252; Merck, Darmstadt, Germany); 10 g of glucose; 5 g of amino acids (casamino acids, vitamin assay; BD, Difco); 1 g of sodium pyruvate; 6 mg of methotrexate sodium salt; nucleosides (100 mg each of adenosine, cytidine, guanosine, thymidine, and uridine); vitamins (5 mg each of biotin, calcium pantothenate, choline, folic acid, inositol, *p*-aminobenzoic acid, thiamine, pyridoxal and nicotinamide, and 2.5 mg of riboflavin); salts ($MgSO_4$ [200 mg], Cl_2K [100 mg], Cl_2Mn [25 mg], NH_4 $(SO_4)_2Fe_6OH_2$ [25 mg], and Cl_2Ca [10 mg]); and buffer (Na_2H PO_4 [8.5 g] and morpholinopropanesulphonic acid hemisodium salt [11 g]).

Several modifications of CAM medium were also tested (Table 1): (i) CAM medium supplemented with cysteine at 50 μg/ml (CAM-Cy medium), (ii) CAM medium in which casamino acids were replaced with a defined mixture containing all the amino acids (including cysteine) present in Eagle's medium for cell culture (1) at 50 μg/ml (AM-Cy medium), and (iii) AM-Cy medium without cysteine (AM medium). In addition, some further culture media, in which other sulphur-containing compounds (cystine at 50 μg/ml, glutathione reduced or oxidized at 250 μg/ml, 2-mercaptoethanol at 100 μg/ml, thioglycolic acid at 500 μg/ml, or dithiothreitol at 500 μg/ml) or reducing compounds (sodium metabisulfite at 200 μg/ml or ascorbic acid at 1,000 μg/ml) were substituted for cysteine in CAM-Cy and AM-Cy media, were also assayed. Media were prepared just before inoculation, sterilized in a boiling water bath for 10 min, and cooled to 48°C before pouring. Glucose, pyruvate, methotrexate, and all reducing compounds were added as filter-sterilized solutions after cooling.

GBS grew in all these media as colonies of similar size to

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TABLE 1. Pigment production by *S. agalactiae* in quasi-defined media

Medium[a]	Components (μg/ml)			Color of GBS colonies
	Casamino acids[a]	L-cysteine[b]	Defined amino acids[c]	
CAM	5,000			White
CAM-Cy	5,000	50		Orange-red
AM			550	White
AM-Cy		50	550	Orange-red

[a] See the text.
[b] Glutathione, dithiothreitol, thioglycolic acid, ascorbic acid, and sodium metabisulfite can substitute for L-cysteine. See the text.
[c] Final concentration in the medium, 50 μg/ml of each of the following amino acids: L-arginine, L-histidine, L-isoleucine, L-leucine, L-lysine HCl, L-methionine, L-phenylalanine, L-threonine, L-tryptophan, L-tyrosine, and L-valine.

those grown in blood agar (1 to 1.5 mm). In CAM and AM media (both without cysteine), colonies were white, and in CAM-Cy and AM-Cy media, GBS colonies were orange-red (pigment intensity, 2+, 3+) (Table 1). In the media where cystine, glutathione, 2-mercaptoethanol, thioglycolic acid, or dithiothreitol substituted for cysteine, GBS grew as orange-red colonies (pigment intensity, 2+, 3+). When sodium metabisulfite or ascorbic acid substituted for cysteine, GBS also grew as orange-red colonies, but pigment production was weaker (1+, 2+). All three GBS strains used to develop these media grew with the same characteristics after three successive subcultures in the same medium. And all the additional GBS strains tested grew as white colonies in CAM and AM media and as pigmented colonies in CAM-Cy and AM-Cy media.

GBS has an absolute nutritional requirement for cystine, which could be replaced with cysteine or glutathione but not with thioglycolate or dithiothreitol (6, 7, 18, 20). On the other hand, the effects of cysteine on stimulation of GBS growth from a small inoculum (20) seem related to the redox potential reduction, because other reducing agents, such as thioglycolic and ascorbic acids, can be substituted for cysteine. Regarding GBS pigment, anaerobic incubation in CAM and AM media (both without cysteine) did not lead to pigment production, cystine and oxidized glutathione had an effect similar to that of cysteine or reduced glutathione, and in certain conditions, pigment production does not require anaerobiosis (12). These facts could indicate that the effect of sulphur-containing compounds is not a consequence solely of the reduction of the redox potential of the medium. In addition, the chemical nature of GBS pigment, the biochemical pathway that leads to its synthesis, and its physiological role are not known (19). Because of all that, it is difficult to hypothesize about the role of sulphur-containing compounds and reducing agents in GBS pigment production. There is approximately 800 μg of cystine/ml (in peptides from the albumin digest) in all the media studied, and though this fulfills the nutritional requirements (GBS grow in AM and CAM media without cysteine), there is no effect on pigment production. Perhaps this means that in addition to the active peptide Ile-Ala-Arg-Arg-His-Pro-Tyr-Phe, the presence of sulfur-containing compounds as free small molecules is necessary for GBS pigment production, and when they are not present pigment production requires reducing agents. How these compounds cause this effect is not clear, and further work is necessary to investigate this.

The use of media for identification of GBS based on pigment is a promising approach to its detection in clinical samples (3), e.g., Granada medium, which is now marketed in the United States (Hardy Diagnostics, Santa Maria, Calif.) and in the European Union (Biomedics, Madrid, Spain). These media are empirically designed, and knowledge of the role that sulphur-containing compounds play in production of pigment and availability of defined media for GBS pigment production could help to improve them.

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REFERENCES

1. **Eagle, H.** 1959. Amino acid metabolism in mammalian cell culture. Science **130:**432–437.
2. **Islam, A. K. M.** 1977. Rapid recognition of group B streptococci. Lancet **i:**256–257.
3. **Koneman, E. W., D. D. Allen, W. M. Janda, P. C. Schreckenberger, and W. C. Winn.** 1997. Gram positive cocci, p. 608. *In* Color atlas and textbook of diagnostic microbiology, 5th ed. Lippincott, Philadelphia, Pa.
4. **Merritt, K., and J. N. Jacobs.** 1976. Improved medium for detecting pigment production by group B streptococci. J. Clin. Microbiol. **4:**379–380.
5. **Merritt, K., and J. N. Jacobs.** 1978. Characterization and incidence of pigment production by human clinical group B streptococci. J. Clin. Microbiol. **8:**105–107.
6. **Mickelson, M. N.** 1976. Effects of nutritional characteristics of *Streptococcus agalactiae* on inhibition of growth by lactoperoxidase-thiocyanate-hydrogen peroxide in chemically defined medium. Appl. Environ. Microbiol. **32:**238–244.
7. **Milligan, T. W., T. I. Doran, D. C. Straus, and S. J. Mattingly.** 1978. Growth and amino acid requirements of various strains of group B streptococci. J. Clin. Microbiol. **7:**28–33.
8. **Rosa, M., D. Villarreal, D. Vega, C. Miranda, and A. Martinez-Brocal.** 1983. Granada medium for detection and identification of group B streptococci. J. Clin. Microbiol. **18:**779–785.
9. **Rosa, M., M. Perez, C. Carazo, L. Pareja, J. I. Peis, and F. Hernández.** 1992. New Granada medium for detection and identification of group B streptococci. J. Clin. Microbiol. **30:**1019–1021.
10. **Rosa-Fraile, M., A. Sampedro, A. Ruiz-Bravo, S. Sanbonmatsu, and G. Gimenez-Gallego.** 1996. Identification of serum and urine proteins responsible for enhanced pigment production by group B streptococci as amylases. Clin. Diagn. Lab. Immunol. **3:**594–596.
11. **Rosa-Fraile, M., A. Sampedro, J. Varela, M. García-Peña, and G. Gimenez-Gallego.** 1999. Identification of a peptide from mammal albumins responsible for enhanced pigment production by group B streptococci. Clin. Diagn. Lab. Immunol. **6:**425–426.
12. **Rosa-Fraile, M., J. Rodriguez-Granger, M. Cueto-López, A. Sampedro, E. Biel Gaye, J. M. Haro, and A. Andreu.** 1999. Use of Granada medium to detect group B streptococcal colonization in pregnant women. J. Clin. Microbiol. **37:**2674– 2677.
13. **Ruoff, K. L., R. A. Whiley, and D. Beighton.** 1999. *Streptococcus*, p. 283– 296. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 7th ed. American Society for Microbiology, Washington, D. C.
14. **Schrag, S. J., S. Zywicki, M. M. Farley, A. L. Reingold, L. E. Harrison, L. B. Lefkowitz, J. L. Hadler, M. Danila, P. R. Cieslak, and A. Schuchat.** 2000. Group B streptococcal disease in the era of intrapartum antibiotic prophylaxis. N. Engl. J. Med. **342:**15–20.
15. **Spellerberg, B., B. Pohl, G. Haase, S. Martin, J. Weber-Heynemann, and R. Lutticken.** 1999. Identification of genetic determinants for the haemolytic activity of *Streptococcus agalactiae* by ISS1 transposition. J. Bacteriol. **181:** 3212–3219.
16. **Tapsall, J. W.** 1986. Pigment production by Lancefield-group B streptococci (*Streptococcus agalactiae*). J. Med. Microbiol. **21:**75–81.
17. **Tapsall, J. W.** 1987. Relationship between pigment production and haemolysin formation by Lancefield group B streptococci. J. Med. Microbiol. **24:** 83–87.
18. **Terleckyj, B., N. P. Willet, and G. D. Shockman.** 1975. Growth of several cariogenic strains of oral streptococci in a chemically defined medium. Infect. Inmun. **11:**649–655.
19. **Wennestrom, D. E., L. N. Lee, A. G. Baseman, D. J. LeBlanc, C. E. Cerniglia, and K. M. Trotter.** 1991. Genetics and characterization of group B streptococcal pigment, p. 224. *In* G. M. Dunny, P. P. Cleary, and L. L. McKay (ed.), Genetics and molecular biology of Streptococcus, Lactococcus, and Enterococci. American Society for Microbiology, Washington, D.C.
20. **Willet, N. P., and G. E. Morse.** 1966. Long chain fatty acid inhibition of growth of *Streptococcus agalactiae* in a chemically defined medium. J. Bacteriol. **91:**2245– 2250.