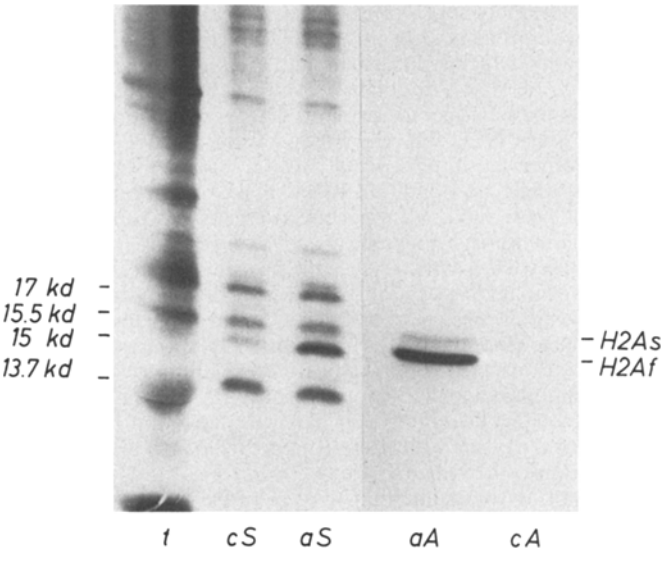


Fluorography of histone H2A antigens after radioimmunoprecipitation. Total RNA (10 µg) of *Sciara coprophila* embryos was translated in vitro and translation products were labeled with ^{35}S methionine [6]. An aliquot was taken for analysis of total translation products (lane t). The remainder was incubated with the monoclonal antibody By 187 directed against *Drosophila melanogaster* histone H2A (lanes a) [7], or a control monoclonal antibody which showed no specific crossreactivity with *Sciara* proteins (lanes c), and immunocomplexes were precipitated with fixed *Staphylococcus aureus* cells (10 mg) (lanes S) or with protein A-conjugated sepharose beads (2 mg, Pharmacia, Uppsala, Sweden) (lanes A) and washed five times in washing solution (1% Nonidet P40, 0.01% deoxycholic acid, 0.01% SDS, 5 mM potassium iodide, 2 mM methionine in PBS). Isolated radiolabeled protein fractions were separated by SDS-polyacrylamide gel electrophoresis (15% acrylamide) and bands were visualized by fluorography as described [6]. Molecular weights and positions of the H2A antigens are indicated.

absence of specific antibodies, causing problems in the detection of specific proteins present in small amounts. Platt et al. [5] tried to overcome these difficulties by a complicated and elaborate modification of the precipitation technique, accepting lower recoveries of the radiolabeled antigens.
In our recent work [6] we analyzed radiolabeled histone H2A in vitro translation products of total RNA of *Sciara coprophila* embryos using a monoclonal antibody directed against *Drosophila melanogaster* histone H2A. Here, in similar radioimmunoprecipitation experiments we compare, after fluorography, results obtained by precipitation of the histones H2A-anti-H2A antibody complexes either with protein A-containing *St. aureus* cells (fig. lane aS) or with protein A-conjugated sepharose (lane aA). As a control we repeated the precipitation experiment using a monoclonal antibody which showed no specific reaction with *Sc. coprophila* proteins (lane cS, precipitated with *St. aureus,* and lane cA, precipitated with protein A-conjugated sepharose). Total radiolabeled translation products are shown in lane t. As can be seen, specific H2A antigens (H2As and H2Af) are selectively precipitated by protein A-conjugated sepharose and in the presence of the H2A specific antibody (compare lane aA with lane cA). In contrast, fixed *St. aureus* cells (lanes aS and cS) precipitate not only these H2A antigens, but also additional proteins non-specifically (compare lane aS with cS). These might, as judged by their electrophoretic mobility [6], correspond to the other core histones. With these latter experiments specific H2A antigens cannot be reliably analyzed. The unspecific binding of core histones could result from the interaction of the histones with the bacterial DNA in fixed staphylococcal cells.
As a consequence, in performing radioimmunoprecipitation experiments with histone antigens, protein A-conjugated sepharose should be used as an immunoadsorbent, since this technique displays high adsorption capacity, rapid binding, and advantageous sedimentation properties, but prevents unspecific binding of other proteins and low recovery of antigens.

Acknowledgment. The support of Professor Dr Seyffert is gratefully acknowledged.

1 Forsgren, A., and Sjöquist, J., J. Immun. *97* (1966) 822.
2 Forsgren, A., and Sjöquist, J., J. Immun. *99* (1967) 19.
3 Brunda, M. J., Minden, P., Sharpton, T. R., McClatchy, J. K., and Farr, R. S., J. Immun. *119* (1977) 193.
4 Ivarie, R. D., and Jones, P. P., Analyt. Biochem. *97* (1979) 24.
5 Platt, E. J., Karlsen, K., Lopez-Valdivieso, A., Cook, P. W., and Firestone, G. L., Analyt. Biochem. *156* (1986) 126.
6 Ruder, F. J., Frasch, M., Mettenleiter, T. C., and Büsen, W., Devl Biol. *122* (1987) 568.
7 Frasch, M., Thesis, University of Tübingen, 1985.

0014-4754/88/040347-02$1.50 + 0.20/0

Reversion of *Lactobacillus lactis* protoplasts

P. Bican and A. Spahni [1]

Swiss Federal Dairy Research Institute, CH-3097 Liebefeld-Bern (Switzerland)
Received 21 September 1987; accepted 23 December 1987

Summary. More than 99% of *L. lactis* cells have been converted to protoplasts upon digestion of cell walls with mutanolysin (N-(acetyl)muramidase). Functional protoplasts were obtained even with the lowest level of the enzyme that was used (0.1 $U \cdot ml^{-1}$ of the cell suspension) and after incubation at 37 °C for 2 min. The regeneration of the polymerized cell wall appears to be induced by a cell homogenate of the same organism.
Key words. L. lactis; protoplasts; protoplast regeneration.

Contrary to other species of lactic acid bacteria [2] *L. lactis,* which plays an important role in food science and technology has attracted little attention in genetic and biosynthetic studies. The degradation of the cell walls and membrane polymers of Gram-positive bacilli is facilitated by the fact that these organisms possess only one membrane system. The supramolecular aggregates of the bacterial surface can be dissociated by muralytic enzymes, e.g. mutanolysin or

lysozyme[3,4]. The resulting structures are protoplasts; the outer part of these represents a barrier, comparable in its orderly arrangement with the cytoplasmic membrane. There are close relationships between genetic interactions and the physical state of the protoplast membrane, and membrane-bound functions[5].

Studies have been carried out to elucidate modifications in the composition of cell envelopes in order to clarify the processes of protoplast fusion and regeneration, and to investigate the role of protoplasts in transformation and gene cloning experiments[5-11].

The primary purpose of this communication is to describe conditions for conversion of *L. lactis* to protoplasts which possess the specific functions of the intact vegetative cell necessary for adequate regeneration.

Materials and methods. The parent culture of *L. lactis* 157.5 (our Institute's stock culture collection) was maintained in glycerol at − 20 °C. The strain was propagated for 24 h at 37 °C in Rogosa's LCM-medium[12]. The passage was for 15 h in LCM with 2% glucose, then for 6 h in LCM with 1% glucose. Exponentially growing cells were collected at 3600 × g, 5 min, 4 °C. The sedimented cells were resuspended in a solution containing 20 mM Hepes protoplast buffer (HPB, pH 7.0), 1 mM $MgCl_2$, 0.5% gelatine, and 0.3 M lactose. The conversion of the cultured cells into a form sensitive to osmotic pressure was accomplished by enzymic treatment of cells adjusted to 0.8 A_{600} in HPB with mutanolysin (N-(acetyl)muramidase – Sigma, St. Louis, MO, USA) at 1 $U \cdot ml^{-1}$, for 30 min at 37 °C. Protoplast formation was monitored by phase contrast microscopy.

Cell lysates were prepared under sterile conditions in an adapted Sorvall Omni Mixer (plastic propeller) by mechanical breakage of the cells with glass beads (0.10–0.11 mm). The proportions used were 2.5 $g \cdot ml^{-1}$ of 0.8 A_{600} suspension, with 8 × 30-s breakage cycles and 30-s cooling intervals. The temperature was controlled by a cold ethanol bath (− 20 °C)[13].

Details of the regeneration procedure were close to those described by Lee-Wickner and Chassy[14], but with some modifications. The summarized protocol is given in figure 2. Lactose (0.3 M) as osmotic stabilizer, 0.1 M N-acetylglucosamine and cell lysate (0.1 $ml \cdot ml^{-1}$ of regeneration medium) were used. The plates were cultivated under anaerobic conditions at 37 °C using a BBL Gas Pak System (Cockeysville, MD, USA).

The samples for electron microscopy were prepared by means of the rapid cryofixation procedure[15].

Results and discussion. Mutanolysin concentrations, ranging from 0.1 to 100 $U \cdot ml^{-1}$ of the suspension at the employed cell density were examined. Even at the lowest level of the enzyme and with the shortest incubation at 37 °C (2 min), an extensive degradation of the outer surface of *L. lactis* was observed. With some *L. lactis* strains the components of the buffer, without mutanolysin, destabilized the surface of the cells after long incubation (> 10 h, at 37 °C), giving rise to some protoplasts. This observation is difficult to explain. The cell wall preparations of these osmotically sensitive strains were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The profiles obtained were compared with the electrophoretic behavior of surface proteins of *L. lactis* 157.5. In spite of the relatedness revealed by the gels, in the case of sensitive strains some protein bands were absent or considerably diminished (pictures not shown). Although these constitute a small percentage of a sample, the differences may be indicative of an unstable surface. Additional work is needed to clarify this.

When viewed in the electron microscope, *L. lactis* exhibits an ultrastructure typical for a Gram-positive organism. The micrograph of the whole cell (fig. 1 A) shows a bilaminar arrangement corresponding to the cell wall and cytoplasmic membrane. When the wall was degraded by mutanolysin, the polypeptides of the peptidoglycan layer were selectively released from the cytoplasmic membrane (fig. 1 B).

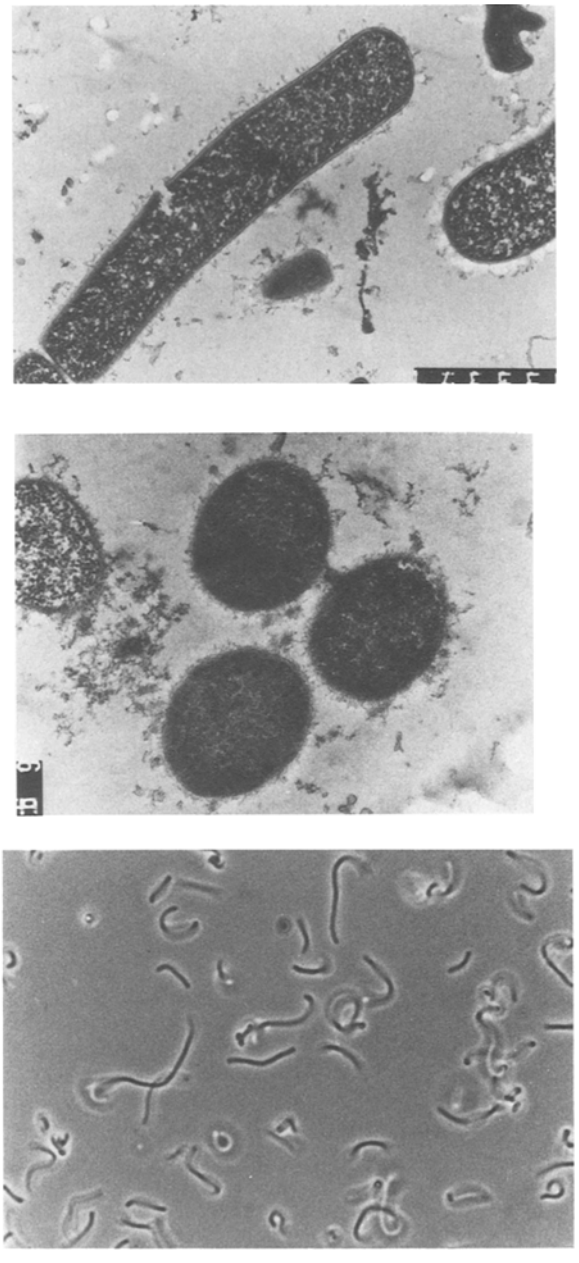
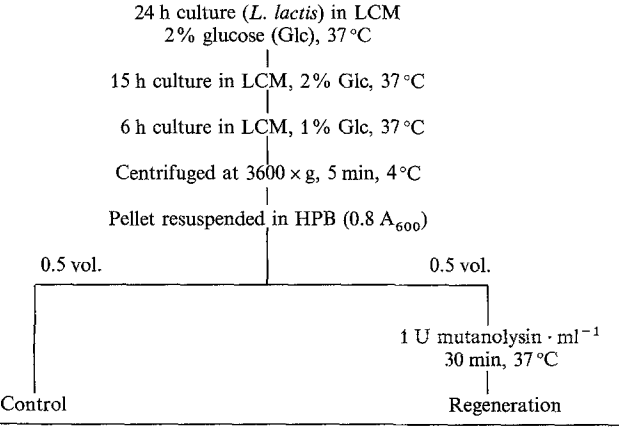


Figure 1. **A** Electron micrograph of *L. lactis;* **B** Protoplasts of *L. lactis;* **C** Phase contrast micrograph of the regenerated cells.

In our previous experiments, we tried to terminate the effect of mutanolysin and thus prepare spherical cell forms either with wall residues or already as protoplasts, showing little structural modification. Although only a minimal concentration of the enzyme was required to bring about enzymic digestion of the cell wall macromolecules, we did not succeed in restricting the enzyme-catalyzed reaction by washing the protoplast suspension with isotonic buffers immediately after protoplast-formation was completed. Attempts have also been made to use lysozyme (in the range of 20–200 $\mu g \cdot ml^{-1}$ of the cell suspension, at 37 °C over a period of 180 min) for hydrolysis of the cell wall of *L. lactis*. Neither high concentration, nor long incubation induced significant changes except for some perturbation of the envelope components. The



(C_{lac})	(C)	(P_r)	(O_{rc})
LCM, 1% Glc	LCM, 1% Glc	LCM, 1% Glc	LCM, 1% Glc
0.3 M lactose	–	0.3 M lactose	–
0.1 ml of $10^{-5} - 10^{-7}$ dilutions	0.1 ml of $10^{-5} - 10^{-7}$ dilutions	0.1 ml of $10^{-2} - 10^{-6}$ dilutions	0.1 ml of $10^{-2} - 10^{-6}$ dilutions
	10 ml LCM, 1% Glc, soft agar (0,5%) overlay		
0.3 M lactose	–	0.3 M lactose	–
	1 ml cell homogenate bottom agar (1.5%), 1% Glc		
0.3 M lactose	–	0.3 M lactose	–
	10 plates per dilution anaerobic conditions, 37 °C		

C_{lac}, Control with lactose; C, Control without lactose; P_r, Protoplast regeneration; O_{rc}, Osmotic resistant cells. Counting of colonies: after 4, 11, and 18 days. Total colonies (TC) = $\frac{C_{lac} + C}{2}$; Protoplasts = TC − O_{rc}; Regeneration = $P_r - O_{rc}$.

Figure 2. Outline of the regeneration procedure.

Expt	C_{lac}[a, b]	C[b]	P_r[b]	O_{rc}[b]	TC[b]	Protopl.[b]	Regener. (%)
1	9.86×10^8	9.62×10^8	2.87×10^7	2.65×10^7	9.74×10^8	9.48×10^8	0.24
2	3.38×10^8	3.56×10^8	3.03×10^5	1.00×10^3	3.47×10^8	3.47×10^8	0.09
3	3.81×10^8	9.30×10^8	2.30×10^7	1.91×10^7	6.56×10^8	6.37×10^8	0.62
4	6.07×10^8	8.32×10^8	3.11×10^7	2.36×10^7	7.20×10^8	7.09×10^8	1.05

[a] see fig. 2 for abbreviations and calculation; [b] CFU/ml.

yield of protoplasts, which were irregular and defective in form, was low.

The regeneration frequencies were calculated as proposed by Lee-Wickner and Chassy [14] and Chen et al. [16]. Our replicate experiments gave results ranging from 0.1 to 1%. Typical results are presented in the table.

To facilitate the easy conversion of protoplasts from *C. pasteurianum*, Minton and Morris [17] enriched their regeneration medium with N-acetylglucosamine, a cell wall component. Clive and Landman [18] reported an increase of regeneration of *B. subtilis* protoplasts by adding isolated cell walls. Unfortunately, in our experiments killed cells or cell walls alone did not promote the regeneration of protoplasts. Instead, the lysed, homogenized cells of the same organism and incorporation of N-acetylglucosamine proved to be useful. There are two explanations that would account for the mode of action of this supplement to the growth medium. First, its constituents might function as precursors of cell wall polymers. Second, metabolic activity of the cytoplasmic membrane appears to be induced.

Differences in the capacity to regenerate protoplasts depend upon the composition and functions of the cell envelope components, which may be specific for one species. Therefore, regeneration procedures cannot be considered to be of general application.

1 The authors thank Dr M. Müller, Laboratory for Electron Microscopy I, Swiss Federal Institute of Technology, Zürich for generous help in the procedures involving electron microscopy and Dr S. K. Gupta of the Swiss Federal Institute for Agricultural Chemistry and Hygiene of Environment, Liebefeld-Bern for critically reading the manuscript.

2 McKay, L. L., Antonie van Leeuwenhook *49* (1983) 259.
3 Kaback, H. R., Meth. Enzymol. *22* (1976) 99.
4 Yokogawa, K., Kawata, S., Nishimura, S., Ikeda, Y., and Yoshimura, Y., Antimicrob. Ag. Chemother. *6* (1974) 156.
5 Hopwood, D. A., A. Rev. Microbiol. *35* (1981) 237.
6 Rogers, H. J., Ward, J. B., and Burdett, I. D. J., XXVIII Symp. Soc. gen. Microbiol., Univ. Southhampton, p. 139. Cambridge Univ. Press, London 1978.
7 Ward, J. B., XXVIII Symp. Soc. gen. Microbiol., Univ. Southhampton, p. 249. Cambridge Univ. Press, London 1978.
8 Wyrick, P. B., and Rogers, H. J., J. Bact. *116* (1973) 456.
9 Schaeffer, P., Cami, B., and Hotchkiss, R. D., Proc. natl Acad. Sci. USA *73* (1976) 2151.
10 Gasson, M. J., FEMS Microbiol. Lett. *9* (1980) 99.
11 Klaenhammer, T. R., Curr. Microbiol. *10* (1984) 23.

12 Efthymiou, C., and Hansen, P. A., J. infect. Dis. *110* (1962) 258.
13 Casey, M. G., and Meyer, J., J. Dairy Sci. *68* (1985) 3512.
14 Lee-Wickner, L.-J., and Chassy, B. M., Appl. envir. Microbiol. *48* (1984) 994.
15 Mueller, M., Meister, N., and Moor, H., Mikroskopie (Wien) *36* (1980) 129.
16 Chen, W., Ohmiya, K., and Shimizu, S., Appl. envir. Microbiol. *52* (1986) 612.
17 Minton, N. P., and Morris, J. G., J. Bact. *155* (1983) 432.
18 Clive, D., and Landman, O. E., J. gen. Microbiol. *61* (1970) 233.

0014-4754/88/040348-04$1.50 + 0.20/0

Direct demonstration of production of transforming growth factor activity by embryonic chick tissue

J. C. McLachlan, J. Macintyre, D. D. Hume and J. Smith

Department of Biology and Preclinical Medicine, University of St. Andrews, Bute Medical Buildings, St. Andrews, Fife KY16 ODA (Scotland)
Received 4 November 1987; accepted 28 December 1987

Summary. The presence of transforming growth factor activity in early chick embryos was directly demonstrated by the ability of limb and tail buds to induce anchorage independent division in NRK 49 f cells. Colony number increased with limb bud number and developmental stage. Medium conditioned by tail buds contained some stimulating effect, and strongly promoted the action of other transforming growth factors.
Key words. Transforming growth factor; chick embryo; limb bud.

Although it is possible to isolate transforming growth factors [1] from embryos [2-6] it is not yet clear what function they normally fulfil. Two problems arise with the standard procedure. One is that to obtain enough starting material, embryos are usually homogenised, losing information about spatial distribution of the factors. A second is that only known transforming growth factors can be readily identified when present in small amounts, so unknown transforming growth factors may not be detected. An alternative method which avoids both these problems is to test the ability of pieces of embryonic tissue directly for their ability to induce colony formation by fibroblasts grown in soft agar. We selected chick embryo limb buds as the test tissue, since chick embryos are easy to obtain at predetermined stages, and the limb buds can be removed in a consistent manner from embryo to embryo. There is already evidence that limb buds produce mitogenic growth factors [7, 8].

Fertile cross-bred hens eggs (Muirfield Hatcheries, Kinross) were incubated for periods of 4–5 days at 39 °C, then opened and staged by the criteria of Hamilton and Hamburger [9]. Embryos were removed into 30-mm dishes of Hams F12 medium containing 5% foetal calf serum (Gibco). Limb buds were removed by a cut across the proximal margin at the body wall, using electrolytically sharpened tungsten needles. Tail buds were removed by cuts transverse to the neural tube, at a distance equivalent to the width of the tail. Buds were washed once by transferal to fresh medium. Conditioned medium was prepared by culturing groups of 3 tail buds in 0.3 ml serum-free medium for 24 h, after which the medium was removed and made up to 1 ml with serum-free medium.

NRK clone 49f cells (a gift from Dr Austin Smith, Department of Zoology, Oxford) were cultured in Hams F12 containing 5% foetal calf serum (FCS) and passaged at 3–4-day intervals. Equal volumes of 0.6% Bacto-agar (Difco) and 2× Dulbecco's MEM (Flow Laboratories) with 22.4% FCS containing 4×10^3 NRK 49f cells per ml were mixed together at 40 °C. At final concentration the medium also contained 50 IU/ml penicillin and 50 µg/ml streptomycin (both Flow Laboratories). 1 ml of this mixture was added to a 30-mm bacteriological grade plastic petri dish (Sterilin). Limb and tail buds were placed in petri dishes prior to addition of the cells. Buds were transferred using handmade tantalum foil trays. EGF (Sigma), TGF-β (Peninsula Laboratories), conditioned media and control media were added to the dishes in 0.24-ml volumes.

Colony number was assessed 9 days after the start of each experiment. The scoring criterion was that a colony must contain four or more cells, as observed using an inverted microscope. No colonies were observed in controls lacking limb buds or additional growth factors, in controls in which the soft agar had been stirred with forceps previously used to handle embryos, or in controls where yolk particles were added to the dish. However, colonies were observed in the presence of limb buds, and the number of colonies increased with the number of limb buds (fig. 1). 3 tail buds induced colonies at a similar frequency to one limb bud. The effect of developmental stage was investigated by assaying the number of colonies induced by the presence of 2 wing buds per dish, at stages 18–26, and it was found that the number of colonies tended to increase with developmental stage (fig. 2),

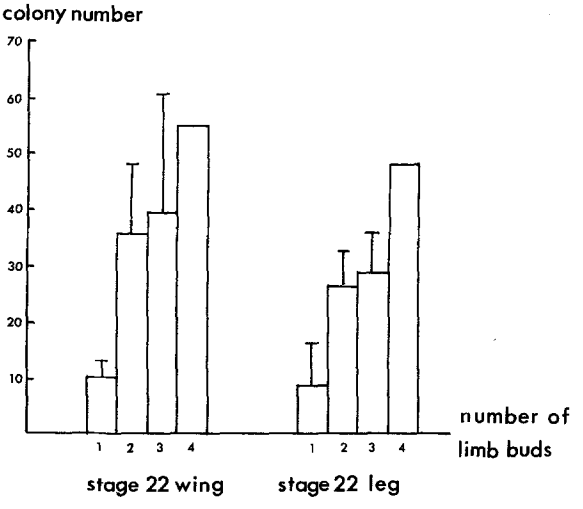


Figure 1. Effect on colony number of increasing number of wing and leg buds. Each value is the mean of between 6 and 8 determinations, except those involving 4 limbs, which were determined once only. Error bars represent standard deviations.