INFECTION AND IMMUNITY, Oct. 1988, p. 2570–2575
0019-9567/88/102570-06$02.00/0

Vol. 56, No. 10

Regulation of Hemolysin Expression in *Actinobacillus pleuropneumoniae* Serotype 1 by Ca^{2+}

JOACHIM FREY* AND JACQUES NICOLET

Institute of Veterinary Bacteriology, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland

Received 18 March 1988/Accepted 21 June 1988

***Actinobacillus pleuropneumoniae*, the causative agent of swine pleuropneumonia, secretes a hemolytic activity which is thought to be a factor involved in the pathogenesis of the disease. The biosynthesis of hemolysin by serotype 1 strain 4074 was strongly dependent on the activity of free Ca^{2+} in the growth medium. At activities of free Ca^{2+} below 50 μM, very low hemolytic activities could be detected in the growth medium and in cell extracts. Maximal hemolytic activities of up to 400 hemolytic units per ml could be measured in growth medium containing free Ca^{2+} activities above 3 mM. Other bivalent cations did not stimulate the production of hemolysin. Neither the growth rate nor the secretion of hemolysin was affected by increasing Ca^{2+} concentrations in the medium. The hemolysin of serotype 1 did not require Ca^{2+} as a cofactor for the lysis of erythrocytes. Ca^{2+} induced the expression of a 105-kilodalton protein, which was secreted. This protein comigrated with purified hemolysin on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and exhibited hemolysin activity upon purification. Inhibition experiments with rifampin suggest that the hemolysin of *A. pleuropneumoniae* is regulated by Ca^{2+} at the transcriptional level. The threshold of hemolysin induction was around 700 μM free Ca^{2+}, a concentration which is similar to that found in blood serum. The Ca^{2+}-inducible hemolysin represents a novel type of positively regulated bacterial gene expression.**

Actinobacillus (*Hemophilus*) *pleuropneumoniae*, a gram-negative bacterium belonging to the family of the *Pasteurellaceae*, causes a severe, contagious, and often fatal respiratory tract disease of pigs, known as porcine pleuropneumonia (25). This disease is of great economical importance where swine production is industrialized, since it causes severe outbreaks principally in fattening units. Transmission of the etiological agent occurs mainly from pig to pig, which is the main host. Surviving of the organism in the environment is possible but is assumed to be of short duration (19). The clinical and pathological effects of both natural and experimental infections are well documented (19, 24). Although the pathogenesis of this disease has not been elucidated completely, experimental studies have revealed that one or more toxins produced by *A. pleuropneumoniae* may be implicated in the disease process (1). Characteristic for *A. pleuropneumoniae* strains is a secreted hemolytic activity, which is thought to play an important role in virulence (12). The degree of virulence of different *A. pleuropneumoniae* strains seems to be related to the net hemolytic activity produced by these strains (J. Nicolet, Ph.D. thesis, University of Berne, Berne, Switzerland, 1970) as well as to the capsular structure and the content of lipopolysaccharides (6, 10). In addition, it is now well documented that hemolysins are involved in virulence of several other bacteria (2, 4, 9).

The nature of *A. pleuropneumoniae* hemolysins is only very poorly understood. It is not clear whether all serotypes produce the same type of hemolysin. *A. pleuropneumoniae* serotypes 1, 2, 3, and 5 were reported to produce heat-labile hemolysins (15, 16; S. Rosendal, J. Devenish, J. I. MacInnes, J. H. Lunsden, S. Watson, and H. Xun, Am. J. Vet. Res., in press). Nakai et al. (18) reported that serotype 2 strains produce a heat-stable hemolysin. The hemolysins of serotypes 1, 2, and 5 were described as RNA-dependent hemolysins by Martin et al. (15). However, Rosendal et al. (in press) described a serotype 1 hemolysin which seemed not to require RNA as a cofactor. We have recently purified the hemolysin of *A. pleuropneumoniae* serotype 1 and characterized it as a 105-kilodalton (kDa) protein (Frey and Nicolet, FEMS Lett., in press).

It has been suggested that the hemolytic activity of *A. pleuropneumoniae* is a function of the medium in which the cells are grown (16). However, the factors that govern the biosynthesis of hemolysin in *A. pleuropneumoniae* remain unknown. To elucidate the role of hemolysin in pathogenesis of *A. pleuropneumoniae* infections, we studied the regulation of hemolysin expression in *A. pleuropneumoniae* serotype 1. In the present study, we have investigated the role of Ca^{2+} in the biosynthesis of this hemolysin. We demonstrate that hemolysin of *A. pleuropneumoniae* serotype 1 is induced by Ca^{2+}. However, Ca^{2+} is not required for the lysis of erythrocytes. In contrast, the biosynthesis of *A. pleuropneumoniae* serotype 2 hemolysin is not induced by Ca^{2+}, but Ca^{2+} strongly enhances erythrocyte lysis.

MATERIALS AND METHODS

Bacterial strains. *A. pleuropneumoniae* type strain 4074 (20), serotype 1 (11), and serotype 2 reference strain 1536 (11) were used.

Growth media, antibiotics, chemicals, and Ca^{2+} concentrations. The following liquid medium was used unless otherwise mentioned: Supplemented Columbia broth: (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 1% IsoVitaleX (BBL) and 10 μg of β-NAD (Sigma Chemical Co., St. Louis, Mo.) per ml. The other media used were S medium (chicken meat extract and chicken serum), described by Kume et al. (13), and RPMI 1640 medium (GIBCO Ltd., Paisley, United Kingdom), referred to as RPMI.

Complementation of the media with Ca^{2+} was made by the addition of $CaCl_2$. Total Ca concentrations of the media were measured by atomic absorption. The chemical activity of Ca^{2+} was measured with a Ca^{2+}-specific electrode (5) and is

* Corresponding author.

referred to herein as "activity of free calcium." Supplemented Columbia broth has a total Ca concentration of 0.6 mM and a free Ca^{2+} activity of 70 μM. Complementation of Columbia broth with $CaCl_2$ concentrations of 0.6, 5, 10, and 20 mM resulted free Ca^{2+} activities of 0.25, 1.35, 3.2, and 6.7 mM, respectively. The free Ca^{2+} in the medium was chelated with EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], a specific Ca^{2+} chelator.

For solid media we used chocolate agar and Columbia agar (Oxoid, Basingstoke, United Kingdom) supplemented with 1% IsoVitaleX and 10 μg of NAD per ml.

EGTA was purchased from Sigma. Chloramphenicol and rifampin (both from Sigma) were used at 50 μg/ml (final concentration). $CaCl_2$, $MgCl_2$, $MgSO_4$, $ZnCl_2$, KCl, LiCl, RbCl, and CsCl were purchased from FLUKA, and proteinase K was purchased from Sigma.

Growth conditions. The *A. pleuropneumoniae* strains were grown on supplemented Columbia agar plates at 37°C over night. Supplemented Columbia broth was then inoculated to an A_{650} of 0.1 and incubated with shaking (130 rpm) at 37°C (unless otherwise stated) to an A_{650} of 0.8 (midexponential growth).

Since *A. pleuropneumoniae* does not grow in RPMI medium, cells were grown on solid chocolate agar plates at 37°C overnight, suspended in RPMI to an A_{650} of 0.8, and then incubated for 1 h at 37°C for hemolysin production.

Hemolysin titration. Unless otherwise noted, *A. pleuropneumoniae* cells were grown in liquid medium to an A_{650} of 0.8. The cells were then harvested by centrifugation. For the titration of the hemolytic activity in the supernatant, dilution series of the supernatant were made in 10 mM Tris hydrochloride–0.9% NaCl (pH 7.5). The addition of $CaCl_2$ to this buffer is marked specifically when used. Samples of 1 ml of these dilutions were then mixed with 1 ml of 1% sheep erythrocytes in the same buffer, incubated for 2 h at 37°C, and sedimented at 4°C for at least 15 h. The A_{540} of the supernatants was measured. Negative controls contained equal volumes of buffer or growth medium and erythrocyte suspensions. One hemolytic unit (HU) is defined as the amount of material which lyses 50% of the sheep erythrocytes in 2 ml of a 0.5% suspension under the assay conditions described above. To determine the hemolytic activity in the cell cytoplasm, the harvested cells were suspended in 0.1 volume of 10 mM Tris hydrochloride (pH 7.5), sonicated with a Branson Sonicator (three times for 30 s) by using the microtip, and then centrifuged at 42,000 × *g* for 30 min. The hemolysin was then determined in this supernatant fraction as described above.

All hemolysin activities were calculated back to give the hemolytic units per milliliter of the unconcentrated culture.

Analysis of proteins by polyacrylamide gel electrophoresis. The proteins in the supernatants of the cultures were analyzed as follows. Samples of 10 ml from cultures at a density of 10^8 cells per ml were centrifuged at a relative centrifugal force of 10,000 × *g*. The cell-free supernatants were concentrated 30-fold in a dialysis bag which was covered with polyethylene glycol 6000 at 4°C, and 20-μl samples were analyzed by sodium dodecyl sulfate-polyacrylamide electrophoresis on a 10 to 17% polyacrylamide gradient gel, as described previously (14), with minor modifications. The proteins were stained with Coomassie blue. The hemolysin was purified as described elsewhere (Frey and Nicolet, in press).

Kinetics of hemolysin induction. A culture of *A. pleuropneumoniae* 4074 was grown exponentially in Columbia broth supplemented with 0.3 mM EGTA (resulting in <10 μM free Ca^{2+}. The cells produced only residual amounts of hemolysin in this medium (Table 1, Fig. 1). After three generations, at an A_{650} of 0.4, the culture was divided in four samples. One sample was grown without additions as a control. The three other cultures were induced by the addition of $CaCl_2$ to a final concentration of 10 mM, corresponding to free Ca^{2+} of 3.25 mM. After 5 min, 50 μg of chloramphenicol per ml was added to one sample to inhibit transcription; to another sample 50 μg of rifampin per ml was added to inhibit transcription initiation. All four samples were continuously incubated at 37°C for another 55 min, and samples were taken at different times to measure the hemolytic activities in the supernatants and in the cell extracts.

TABLE 1. Hemolytic activities in supernatants of serotype 1 strain 4074 cultures in various media and influence of Ca^{2+} complexation or complementation

Medium (mM Ca)	Addition	Free Ca^{2+} activity (mM)	Hemolytic activity (HU/ml)
Columbia (0.6)	None	0.07	10
	0.3 mM EGTA	<0.01	1
	10 mM $CaCl_2$	3.25	350
S (1.2)	None	0.25	70
	0.9 mM EGTA	<0.01	1
	10 mM $CaCl_2$	3.25	300
RPMI (2.5)	None	0.7	150
	2.2 mM EGTA	<0.01	<1
	10 mM $CaCl_2$	5.50	350

RESULTS

Ca^{2+} is required for hemolysin production. The influence of the Ca^{2+} content on the activities of hemolysin secreted to the medium by *A. pleuropneumoniae* 4074 grown in three different media is shown in Table 1. The significant differences in extracellular hemolysin production seemed to be related to the differences of calcium contents in these media (Table 1). Formation of complexes of free Ca^{2+} with the specific Ca^{2+} chelator EGTA resulted in a nearly complete loss of hemolysin activity (Table 1), suggesting that the free Ca^{2+} ions and not the total Ca in the medium is involved in hemolysin production. The activity of free Ca^{2+} was therefore measured in the growth media, and the free Ca^{2+} values rather than the total Ca concentrations were used for the following experiments because of the Ca^{2+} chelating effect of components of the growth media.

Complementation of the media with $CaCl_2$ to free Ca^{2+} activities between 3 and 4 mM resulted in a dramatic increase of the hemolysin production by *A. pleuropneumoniae* 4074 in all three media (Table 1). Addition of Ca^{2+} to the supernatants (after growth) during the hemolysin titration did not increase or restore hemolysin activity. On the other hand, no reduction of the hemolytic activity was observed when EGTA was added to the supernatant after the cell growth, i.e., when titration was in the absence of free Ca^{2+}. These experiments showed that free Ca^{2+} ions are required for the hemolysin production in this strain, but that the biological activity of this hemolysin did not require Ca^{2+} as a cofactor. Incubation of the hemolysin-active supernatants for 10 min at 60°C, or treatment with 0.02 mg of proteinase K per ml for 10 min at 37°C resulted in a complete loss of the hemolytic activity, indicating that the Ca^{2+}-induced hemolysin is a protein.

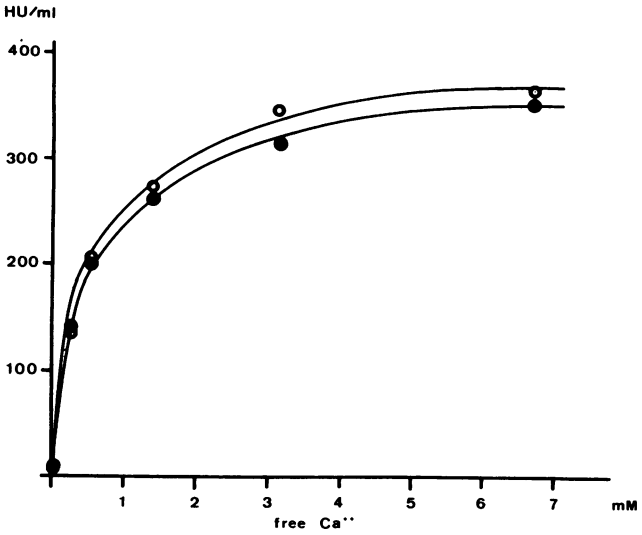

FIG. 1. Dependence of hemolysin production of strain 4074 serotype 1 on free Ca^{2+} in the growth medium. The cells were grown in supplemented Columbia broth with different Ca^{2+} concentrations as described in Materials and Methods. At an A_{650} of 0.8, the hemolysin activities in the supernatants were measured after chelating the Ca^{2+} that was present in the growth medium and titration in buffer containing no Ca^{2+} (○) and titration in buffer containing 10 mM $CaCl_2$ (●) during lysis of erythrocytes. The hemolytic activities are plotted against the activities of free Ca^{2+} in the growth medium.

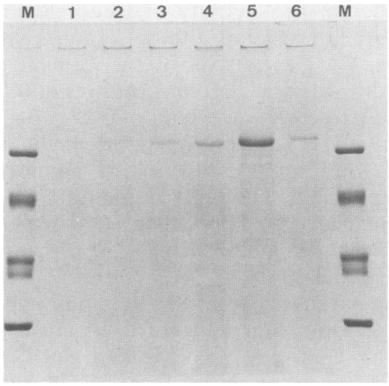

FIG. 2. Increase of the concentration of the 105-kDa hemolysin protein in supernatants of strain 4074 serotype 1 with increasing activities of free Ca^{2+} in the medium. The gel shown was stained with Coomassie blue. The activities of free Ca^{2+} in the media were as follows (lanes): 1, <10 μM (addition of 6 mM EGTA); 2, <10 μM (addition of 1 mM EGTA); 3, 70 μM; 4, 0.25 mM; 5, 3.25 mM. Lane 6 contains purified *A. pleuropneumoniae* strain 4074 hemolysin. The lanes marked M, had the following standards: phosphorylase b (92.5 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa). (Note that the addition of 1 and 6 mM EGTA to the growth medium resulted in free Ca^{2+} <10 μM, which is the lower sensitivity level of our Ca^{2+}-specific electrode.)

One hour of incubation of *A. pleuropneumoniae* 4074 cells suspended in RPMI medium resulted in a hemolytic activity of 150 HU/ml in the supernatant. No cell growth could be observed after 12 h of incubation in this medium. Less than 1 HU of hemolysin per ml in the supernatant was found under the same conditions when RPMI was supplemented with 0.9 mM EGTA, whereas 250 HU/ml was found when 10 mM $CaCl_2$ was added. The addition of 50 μg of chloramphenicol per ml before the addition of Ca^{2+} completely inhibited hemolysin production. This experiment suggests that Ca^{2+} is needed for the biosynthesis of hemolysin and not for its secretion or processing, and that *A. pleuropneumoniae* is able to synthesize proteins in RPMI medium in the absence of growth and cell division.

Expression of hemolysin is specifically induced by Ca^{2+}. To determine whether hemolysin production is induced specifically by Ca^{2+} or whether other ions can be substituted in this process, $CaCl_2$ was replaced in the experiments described in the previous section by $MgCl_2$, $MgSO_4$, $ZnCl_2$, KCl, LiCl, RbCl, or CsCl. None of these salts stimulated hemolysin production. It has to be noted that $ZnCl_2$ had a slightly inhibitory effect on the hemolysin activity.

The extent of induction of hemolysin by Ca^{2+} as well as the optimal Ca^{2+} concentrations for maximum induction were obtained by growing the cells in supplemented Columbia broth with free Ca^{2+} activities ranging from <0.01 to 6.7 mM (Fig. 1). The hemolysin activity in medium with less than 0.01 mM free Ca^{2+} was 2 HU/ml. The maximal induction of hemolysin was about 180-fold when the free Ca^{2+} in the medium was 3 mM or higher (Fig. 1). However, strong induction was evident already at a Ca^{2+} activity of 0.7 mM. Virtually the same results were obtained when the hemolysin activities were measured in the absence of Ca^{2+} or in the presence of 10 mM Ca^{2+} in the assay buffer (Fig. 1). The growth rate (τ) of strain 4074 in supplemented Columbia broth was 40 min and was not dependent on the concentration of $CaCl_2$ added. The number of viable cells per A_{650} unit was also unchanged in the different Ca^{2+}-complemented media. This rules out the possibility that the strong increase of hemolysin activity is a secondary effect of enhanced growth.

The protein analysis on PAGE (Fig. 2) showed that a 105-kDa protein was strongly induced when Ca^{2+} was added to the culture medium. This 105-kDa protein comigrated with purified hemolysin of *A. pleuropneumoniae* (Fig. 2) and exhibited hemolysin activity upon purification (Frey and Nicolet, in press). In addition to the 105-kDa protein, many other protein bands appeared; however, these proteins were at much lower concentrations when the cells were strongly induced with Ca^{2+} (Fig. 2, lanes 4 and 5). A similar effect was observed after strong induction of colicin D with mitomycin c in ColD-harboring strains of *Escherichia coli* (7); this effect could be the result of some cell lysis.

To determine whether Ca^{2+} is involved in the secretory mechanism of the hemolysin, we measured the hemolytic activity of washed and sonicated cells grown in different Ca^{2+} concentrations and compared them with the hemolytic activities of the supernatant. No internal pools of hemolysin were observed at low Ca^{2+} concentrations (Table 2). The internal hemolytic activities increased in parallel with the secreted hemolytic activities dependent upon the level of free Ca^{2+} in the medium. This shows that the strong increase of the hemolytic activity observed by the addition of Ca^{2+} is not due to an enhancement of the hemolysin secretion. The growth temperature did not influence the induction of hemolysin by Ca^{2+}, since very similar hemolysin activities were found in experiments where the cells were grown at 26 and 37°C (Table 2).

Ca^{2+} induces hemolysin expression at the transcriptional level. Figure 3 shows the kinetics of hemolysin induction with Ca^{2+} and the effects of transcription and translation inhibitors. Induction with Ca^{2+} resulted in a significant increase of the hemolytic activity after 5 min. The induction quickly reached steady state, showing a very short lag time

TABLE 2. Intracellular and extracellular hemolysin activities of *A. pleuropneumoniae* serotype 1 strain 4074 grown in supplemented Columbia broth at 37 and 26°C

Free Ca^{2+} (mM)	Growth temp (°C)	Hemolytic activity (HU/ml)	
		Supernatant	Cell extracts
0.07	37	15	0.3
	26	30	0.4
0.25	37	96	3.5
	26		
3.2	37	350	10.0
	26	350	12.0

(less than 1 min). The steady-state kinetic leveled off at hemolysin activities above 250 HU/ml and reached maximum values at 400 HU/ml. The addition of chloramphenicol 5 min after Ca^{2+} induction immediately stopped the increase of hemolysin production, whereas the addition of rifampin resulted first in a slight continuation of the increase in hemolysin production with no further increase after 5 to 10 min. In experiments where chloramphenicol or rifampin was added 5 min before the addition of Ca^{2+}, no induction of hemolysin was detected (results not shown). This shows that the lag time of inhibition of hemolysin production when rifampin (which inhibits initiation of transcription) is added after induction is not due to slow penetration of rifampin. We interpret this delay in inhibition to be due to the half-life of the mRNA of the hemolysin gene. On the other hand, chloramphenicol immediately stopped the hemolysin production due to the action of this antibiotic at the level of translation elongation. Internal hemolysin activities of the cells followed the same pattern as the hemolytic activities measured in the supernatants (but were 30 times lower) and show that the observed effects are not due to secondary effects of secretion.

Regulation of hemolysin expression of serotype 2 strain 1536. To determine whether the Ca^{2+} induction of hemolysin is a common feature in strains of *A. pleuropneumoniae*, we analyzed serotype 2 strain 1536. The hemolysin of this strain is heat labile and sensitive to proteinase K and therefore is assumed to be a protein. Polyclonal antibodies raised against the purified hemolysin of serotype 1 inactivate the hemolytic activity produced by serotype 1 but do not inactivate the hemolytic activity of serotype 2 strain 1536 (results not shown). The hemolysin activity of strain 1536 was independent of the concentration of Ca^{2+} in the growth medium. With Ca^{2+} concentrations of 0, 1, and 5 mM in the titration buffer, the hemolysin activity was 0.2, 1.0, and 3.0 HU/ml regardless of the Ca^{2+} concentration in the growth medium. However, the hemolytic activity in the supernatants of cultures from strain 1536 increased when Ca^{2+} was added during the lysis of erythrocytes. Other bivalent or monovalent cations had no effect on the hemolytic activity of serotype 2 hemolysin. We conclude that the hemolysin of serotype 2 strain is not inducible by Ca^{2+}. However, the hemolysin activity requires Ca^{2+}, probably as cofactor for efficient lysis of erythrocytes. The maximum hemolytic activity produced by serotype 2 was 100-fold lower than that from serotype 1 strain 4074 (Table 1; see above).

DISCUSSION

The aim of this study was to examine the regulation of the expression of the hemolysin of a *A. pleuropneumoniae* serotype 1 strain and to compare it with the hemolysin expression of a serotype 2 strain. The data presented here indicate that the expression of hemolysin of *A. pleuropneumoniae* type strain 4074 (serotype 1) is under the control of exogenous Ca^{2+}. Titration experiments showed that the serotype 1 hemolysin does not require Ca^{2+} for the lysis of the erythrocytes, which we use to assay hemolytic activity. However, this is not a common feature in all *A. pleuropneumoniae* strains. We found that the expression of hemolysin of serotype 2 strain 1536 was not inducible by Ca^{2+} but seemed to need this ion for its activity. It has to be noted that the hemolytic activity under optimal conditions (Table 1; see above) and the virulence of serotype 2 strain 1536 are much lower than those of serotype 1 strain 4074. Experimental infections of swine showed that the lethal dose of serotype 2 is 10 to 100 times higher than that of serotype 1 (Nicolet, Ph.D. thesis). Our experiments provide evidence that both the regulation and the nature of the hemolysin from serotype 1 strain are different from those of serotype 2 strain 1536. We therefore propose to designate the 105-kDa protein hemolysin of strain 4074, which is inducible by Ca^{2+}, as hemolysin type I and the hemolysin of serotype 2, which requires Ca^{2+}

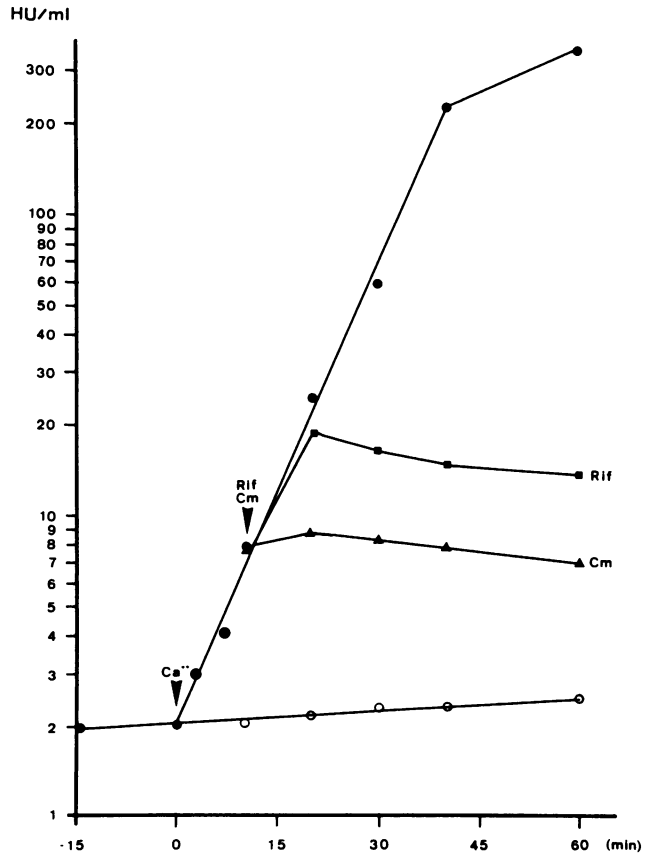

FIG. 3. Kinetics of hemolysin induction with Ca^{2+} and effect of inhibition of translation and transcription after induction. Hemolysin activities measured in supernatants are shown. Symbols: ○, hemolysin activities without Ca^{2+} induction; ●, hemolysin activities after Ca^{2+} induction; ▲, hemolysin activities after addition of chloramphenicol (50 μg/ml, final concentration) 5 min after induction; ■, hemolysin activities after addition of rifampin (50 μg/ml, final concentration) 5 min after induction. The hemolysin activities measured in extracts of washed cells (results not shown) showed the same kinetics but with values 30 times lower than the values of the hemolysin in the supernatants.

as a cofactor, as hemolysin type II. We cannot exclude from our results the possibility that strain 4074 might contain both hemolysin types I and II because of the high hemolytic activity of the type I hemolysin.

We have shown that the hemolysin production of *A. pleuropneumoniae* serotype 1 strain 4074 is induced at the transcriptional level when the external extracellular free Ca^{2+} activity is increased from 50 μM to 3 mM. The exact mechanism of this Ca^{2+} induction, however, remains to be elucidated and requires a profound analysis of the genetic structure of the gene(s) involved in hemolysin production. Calcium ions play a central role in the regulation of various cellular processes in eucaryotic cells. Although little is known about the physiological role of Ca^{2+} in procaryotes, calcium transport systems seem to be very common in bacterial species (23), where calcium is mainly extruded from the cell. The concentration of free Ca^{2+} has recently been determined as 90 nM in *E. coli* cells (8). This value is very similar to the cytosolic concentrations of eucaryotes, and it can be assumed that it would not vary strongly in other gram-negative bacterial cells. Gangola and Rosen (8) also showed that internal free Ca^{2+} is maintained at 90 nM irrespective of the external extracellular Ca^{2+} concentration from 10 μM to 10 mM. However, the internal pool of total calcium increases when external Ca^{2+} concentrations are raised (8). The extracellular Ca^{2+} concentrations that induce hemolysin production in *A. pleuropneumoniae* are approximately 10^4 times higher than the internal concentrations. The response of hemolysin expression in *A. pleuropneumoniae* 4074 to a sudden variation of external Ca^{2+}, however, is very rapid. Ca^{2+} might therefore lay the role of a second messenger and indirectly induce hemolysin expression by stimulating, e.g., a membrane-bound mechanism. It is also possible that Ca^{2+} enters the cell and gets quickly resorbed by a specific protein, which in its turn is activated to induce hemolysin expression (activate or derepress the promoter).

The only procaryotic genes reported up to now that are regulated by extracellular changes in Ca^{2+} concentrations are those of the low-Ca^{2+} response of *Yersinia pestis*, which encodes a set of predominant virulence factors (3, 22, 26). In contrast to the hemolysin operon, gene expression in the low-Ca^{2+} operon of *Y. pestis* is repressed by Ca^{2+} levels above 300 to 1000 μM and requires low Ca^{2+} levels for its expression (21). However, the critical range of the extracellular Ca^{2+} level is very similar to that found in *A. pleuropneumoniae* for the hemolysin induction. It has also to be noted that the induction of hemolysin by Ca^{2+} in *A. pleuropneumoniae* is independent on the growth temperature, which is another difference to the low-Ca^{2+} response of *Y. pestis*.

The pathogenesis of bacterial infections is the result of the concerted action of one or several virulence factors. Knowledge concerning the regulation of the virulence determinants of the parasite is a way to the understanding of the microbial response to its environment. In the case of the low-Ca^{2+} operon of *Y. pestis*, several virulence determinants are expressed at low Ca^{2+} levels, conditions which are found in the intraphagolysosomal environment. Since hemolysin of *A. pleuropneumoniae* is suggested to be a virulence factor, it should be noted that the bacterium finds optimal conditions for hemolysin expression in its natural environment of primary infection, i.e., the interstitial fluid of blood, which has a concentration of free Ca^{2+} of 1.25 mM in vertebrates, corresponding to a Ca^{2+} activity of 700 μM at physiological salt concentrations (17). We have shown (Fig. 2) that the hemolysin induction is nearly complete at a Ca^{2+} activity of 700 μM. This might give the bacterium an advantage in infectivity.

The fact that extracellular Ca^{2+} turns on the gene expression of hemolysin in *A. pleuropneumoniae* serotype 1 represents a new finding in bacterial gene regulation. Its molecular mechanism still has to be elucidated. However, the strong induction rate, the quick response to the inducing agent, and the efficient secretory capacity of the hemolysin not only make this regulated hemolysin gene of *A. pleuropneumoniae* an interesting model in the study of infectivity and virulence but could also provide a useful tool in gene expression systems for biotechnological use.

ACKNOWLEDGMENTS

We are grateful to Mick Chandler, Colin Hughes, and Jos Cox for help and critical advice, to Hans-Jürg Schatzmann for his expertise with the Ca^{2+} determinations, and to Franziska Riesen for skillful technical assistance.

This work was supported by the Swiss National Science Foundation grant 3.570.087.

LITERATURE CITED

1. **Bendixen, P. H., P. E. Shewen, S. Rosendal, and B. Wilkie.** 1981. Toxicity of *Haemophilus pleuropneumoniae* for porcine lung macrophages, peripheral blood monocytes and testicular cells. Infect. Immun. **33**:673–676.
2. **Bernheimer, A. W., and B. Rudy.** 1986. Interactions between membranes and cytolytic peptides. Biochim. Biophys. Acta **864**:123–141.
3. **Brubaker, R. R.** 1983. The Vwa^+ virulence factor of *yersiniae*: the molecular basis of the attendant nutritional requirement for Ca^+. Rev. Infect. Dis. **5**(Suppl.):748–758.
4. **Cavalieri, J. J., G. A. Bohach, and I. S. Snyder.** 1984. *Escherichia coli* α-hemolysin: characteristics and probable role in pathogenicity. Microbiol. Rev. **48**:326–343.
5. **Erne, D., W. E. Morf, S. Arvanitis, Z. Cimerman, D. Amman, and W. Simon.** 1979. Durch elektrisch geladene Ionophore induzierter Ionentransport in Modellmembranen mit Selektivität für Magnesium and Calcium. Helv. Chim. Acta **62**:994–1006.
6. **Fenwick, B. W., B. I. Osburn, and H. J. Olander.** 1986. Isolation and biological characterization of two lipopolysaccharides and a capsular enriched polysaccharide preparation form *Haemophilus pleuropneumoniae*. Am. J. Vet. Res. **47**:1433–1441.
7. **Frey, J., P. Ghersa, P. G. Palacios, and M. Belet.** 1986. Physical and genetic analysis of the ColD plasmid. J. Bacteriol. **166**:15–19.
8. **Gangola, P., and B. P. Rosen.** 1987. Maintenance of intracellular Calcium in *Escherichia coli*. J. Biol. Chem. **262**:12570–12574.
9. **Gill, D. M.** 1982. Bacterial toxins: a table of lethal amounts. Microbiol. Rev. **46**:86–94.
10. **Jensen, A. E., and T. A. Bertram.** 1986. Morphological and biochemical comparison of virulent and avirulent isolates of *Hemophilus pleuropneumoniae* serotype 5. Infect. Immun. **51**: 419–424.
11. **Kilian, M., J. Nicolet, and E. L. Biberstein.** 1978. Biochemical and serological characterization of *Haemophilus pleuropneumoniae* (Matthews and Pattison 1961) Shope 1964 and proposal of a neotype strain. Int. J. Syst. Bacteriol. **28**:20–26.
12. **Kume, K., T. Nakai, and A. Sawata.** 1986. Interaction between heat-stable hemolytic substance from *Hemophilus pleuropneumoniae* and porcine pulmonary macrophages in vitro. Infect. Immun. **51**:563–570.
13. **Kume, K., A. Sawata, and Y. Nakase.** 1978. *Hemophilus* infections in chickens. I. Characterization of *Hemophilus paragallinarum* isolated from chickens affected with coryza. Jpn. J. Vet. Sci. **40**:65–73.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
15. **Martin, P. G., P. Lachance, and D. F. Niven.** 1985. Production of

RNA-dependent hemolysin by *Hemophilus pleuropneumoniae* Can. J. Microbiol. **31**:456–462.

16. **Maudsley, J. R., and S. Kadis.** 1986. Growth and hemolysin production by *Hemophilus pleuropneumoniae* cultivated in a chemically defined medium. Can. J. Microbiol. **32**:801–805.
17. **Moore, E. W., and J. M. Ross.** 1965. NaCl and $CaCl_2$ activity coefficients in mixed aqueous solutions. J. Appl. Physiol. **20**: 1332–1336.
18. **Nakai, T., A. Sawata, and K. Kume.** 1983. Characterization of the hemolysin produced by *Hemophilus pleuropneumoniae*. Am. J. Vet. Res. **44**:344–347.
19. **Nicolet, J.** 1986. *Haemophilus* infections, p. 426–436. *In* A. D. Leman, B. Straw, R. D. Glock, W. L. Mengeling, R. H. C. Penny, and E. Scholl (ed.), Diseases of swine. Iowa State University Press, Ames.
20. **Pohl, S., H. U. Bertschinger, W. Frederiksen, and W. Mannheim.** 1983. Transfer of *Haemophilus pleuropneumoniae* and the *Pasteurella haemolytica*-like organism causing porcine necrotic pleuropneumonia to the genus *Actinobacillus (Actinobacillus pleuropneumoniae* com. nov.) on the basis of phenotypic and deoxyribonucleic acid relatedness. Int. J. Syst. Bacteriol. **33**:510–514.
21. **Pollack, C., S. C. Straley, and M. S. Klempner.** 1987. Probing the phagolysosomal environment of human macrophages with a Ca^{++}-responsive operon fusion in *Yersinia pestis*. Nature (London) **322**:834–836.
22. **Portnoy, D. A., H. F. Blank, D. T. Kingsbury, and S. Falkow.** 1983. Genetic analysis of essential plasmid determinants of pathogenicity in *Yersinia pestis*. J. Infect. Dis. **148**:297–304.
23. **Rosen, B. P.** 1987. Bacterial calcium transport. Biochim. Biophys. Acta **906**:101–110.
24. **Sebunya, T. N. K., and J. R. Saunders.** 1983. *Hemophilus pleuropneumoniae* infection in swine: a review. J. Am. Vet. Med. Assoc. **182**:1331–1337.
25. **Shope, R. E.** 1964. Porcine contagious pleuropneumonia. I. Experimental transmission, etiology and pathology. J. Exp. Med. **119**:357–368.
26. **Straley, S. C., and W. Bowmer.** 1986. Virulence genes regulated at the transcriptional lever by Ca^{2+} in *Yersinia pestis* include structural genes for outer membrane proteins. Infect. Immun. **51**:445–454.