Analytical Biochemistry 397 (2010) 253–255

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Notes & Tips

Chelex-based DNA isolation procedure for the identification of microbial communities of eggshell surfaces

Antonio Manuel Martín-Platero [a,1], Juan Manuel Peralta-Sánchez [b,1], Juan José Soler [b], Manuel Martínez-Bueno [a,*]

[a] Departamento de Microbiología, Facultad de Ciencias, Universidad de Granada, 18071 Granada, Spain
[b] Departamento de Ecología Funcional, Estación Experimental de Zonas Áridas, CSIC, 41003 Almería, Spain

ARTICLE INFO

Article history:
Received 25 September 2009
Received in revised form 26 October 2009
Accepted 29 October 2009
Available online 1 November 2009

ABSTRACT

Microbial communities present on eggshell surfaces of wild birds are weakly studied, especially their influence on embryo infection and, thus, egg viability. Bacterial density of wild bird eggshells is very low, and most DNA extraction protocols are frequently unsuccessful. We have efficiently adapted a chelex-based DNA isolation method for 16S ribosomal gene amplification from the total communities of eggshell surfaces from six avian species.

The eggshell microbiota has been studied mainly on farm eggs using traditional culturing microbiological methods [1]. The emergence of culture-independent methods in microbial diversity studies and their functionality within complex ecosystems explores both cultivable and noncultivable bacteria, that is, a much higher number and diversity [2,3]. The first culture-independent study for these communities was recently published by Shawkey and coworkers [4] on eggs of the avian species *Margarops fuscatus*. DNA analysis is the basis for most of these techniques; consequently, we aimed to achieve a DNA isolation procedure suitable for a high number of samples and total community DNA suitable for further analyses. To this end, we adapted a chelex-based DNA isolation method for studying microbial communities from eggshells. Chelex is a chelating resin that has high affinity for metals ions, protecting the DNA and improving polymerase chain reaction (PCR)[2] [5]. The advantages of using this method are based on the simplicity of the method, its low cost, the avoidance of using hazardous reagents, and the successful PCR from micro amounts of DNA [6,7]. Isolation of DNA by a chelex method has been applied to samples of different origins, including bacteria [8,9] but has limited application to the study of complex communities. In the current work, we have successfully applied a modified chelex method for DNA isolation from eggshells that could be used to study the microbial diversity from this environment.

Samples from 25 clutches (Table 1) were collected in the field with sterile gloves as follows. The eggs were completely cleaned with a sterile swab slightly wet with sterile phosphate buffer and were stocked up in a microcentrifuge tube with 1.2 ml of sterile phosphate solution. Samples were stored at 4 °C until processing. Then 10 μl of each sample was 10:1 serially diluted in sterile saline solution (0.8% NaCl) up to 10^{-6}, and a 100-μl aliquot of each dilution was spread on agar plates for bacterial enumeration on general and selective media: Tryptic Soy Agar (TSA), Hecktoen Enteric Agar (HK), Vogel–Johnsson Agar (VJ), and Kenner Fecal Agar (KF). Plates were incubated at 37 °C for 3 days.

The remaining volume of each sample was retained for total genomic DNA isolation. Bacterial cells were harvested by several centrifugation steps (Fig. 1A) to maximize cell recovery. The swab was placed into a 0.5-ml microcentrifuge tube (top and bottom nicked), which was placed in a 1.5-ml microcentrifuge tube (top nicked) and centrifuged for 5 min at 14,000 rpm. The swab was then discarded, and after cell resuspension the filtrate was returned to the original tube with the remaining sample. Once the cells from the swab were recovered, the phosphate solution containing the bacterial cells was centrifuged for 5 min at 14,000 rpm. The supernatant was discarded, and the cell pellet was submitted to DNA extraction.

DNA isolation (Fig. 1B) consisted of suspending the bacterial pellet in 100 μl of 0.1× TE buffer with 10 mg/ml lysozyme and incubating for 45 min at 37 °C. After this period, 1 μl of 10 mg/ml proteinase K and 1 μl of 10% sodium dodecyl sulfate (SDS) were added and incubated for a further 30 min at 37 °C. Then 100 μl of 10% chelex 100 (200–400 mesh, Bio-Rad) was added, gently mixed,

* Corresponding author. Fax: +34 958 249486.
E-mail address: mmartine@ugr.es (M. Martínez-Bueno).

[1] These authors contributed equally to this work.

[2] *Abbreviations used:* PCR, polymerase chain reaction; TSA, Tryptic Soy Agar; HK, Hecktoen Enteric Agar; VJ, Vogel–Johnsson Agar; KF, Kenner Fecal Agar; SDS, sodium dodecyl sulfate; TTGE, temporal temperature gradient gel electrophoresis; CFU, colony-forming units; BSA, bovine serum albumin.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.10.041

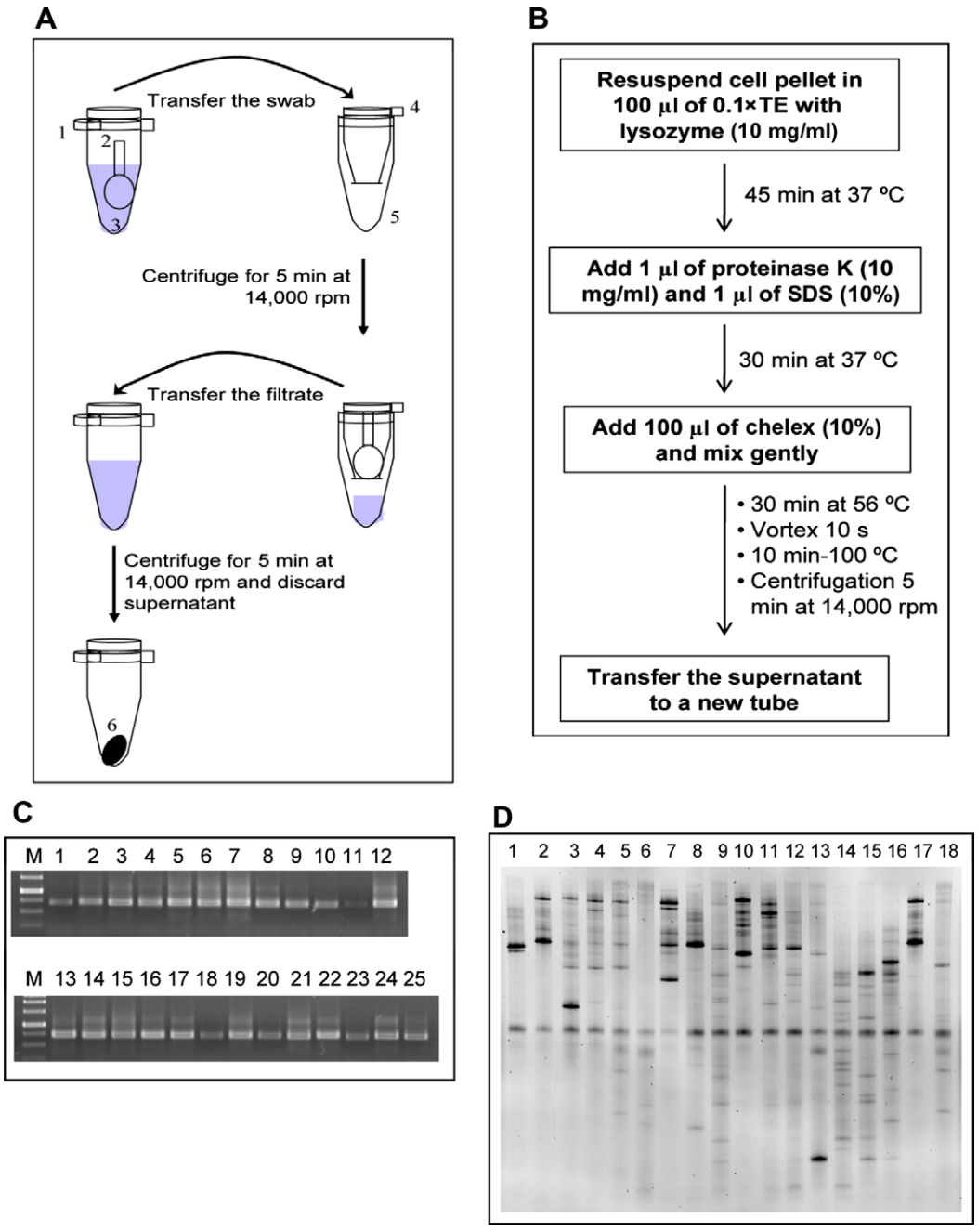

Fig. 1. DNA isolation flow chart. (A) Cell recovery from samples. 1, microcentrifuge tube of 1.5 ml; 2, swab with most of the stick nicked; 3, phosphate buffer; 4, microcentrifuge tube of 0.5 ml with the top and bottom nicked; 5, microcentrifuge tube of 1.5 ml with the top nicked; 6, cell pellet. (B) DNA isolation procedure. (C) Eubacterial 16S ribosomal RNA gene amplification. Lane M, 1-Kb ladder (Biotools); lane 1, P25.2; lane 2, Cast. Quem.2; lane 3, H789.1; lane 4, C.44.1; lane 5, H851; lane 6, BEC 15.1; lane 7, H.HV14.1; lane 8, TEJ 25.1; lane 9, CA51.2; lane 10, C18.1; lane 11, CA61.1; lane 12, 769.1; lane 13, 133.1; lane 14, CA51.1; lane 15, 842.1; lane 16, 55.1; lane 17, N29.1; lane 18, N32.1c; lane 19, HUE 1.1; lane 20, HUE 31.1; lane 21, HUE 27.1; lane 22, HUE 39.1; lane 23, N7.1; lane 24, N32.1; lane 25, N3.1. (D) TTGE profiles of V3 16S ribosomal DNA fragments. Lane 1, Cast. Quem.2; lane 2, H789.1; lane 3, H851; lane 4, H.HV14.1; lane 5, TEJ 25.1; lane 6, CA51.2; lane 7, CA51.1; lane 8, BEC 15.1; lane 9, 769.1; lane 10, 133.1; lane 11, 842.1; lane 12, HUE 1.1; lane 13, HUE 27.1; lane 14, HUE 39.1; lane 15, N29.1; lane 16, N32.1; lane 17, N3.1; lane 18, N32.1c.

and incubated for 30 min at 56 °C. Subsequently, samples were vortexed for 10 s and incubated for 10 min at 100 °C. Finally, samples were centrifuged for 5 min at 14,000 rpm, and the supernatant was transferred to a new microcentrifuge tube.

DNA quality was tested by PCR amplification of the 16S ribosomal DNA gene and temporal temperature gradient gel electrophoresis (TTGE) analysis according to Ogier and coworkers [10]. A negative control was included and consisted of a sterile swab stocked up in a microcentrifuge tube with 1.2 ml of sterile phosphate solution. The control sample was treated as were the rest of the samples, and it did not produce any PCR amplification.

Samples showed bacterial counts for total aerobic mesophilic bacteria from 1.3×10^2 to 3.8×10^9 colony-forming units (CFU)/clutch. Enterobacteriaceae were often at lower levels, with greater heterogeneity counts for enterococci and staphylococci (Table 1). The microbiological groups detected were within the bacterial groups described on eggshells of other avian species [1,11], with densities that differed for different species of birds (J.M. Peralta-Sánchez et al., unpublished).

The DNA isolated by the chelex-based protocol was suitable for PCR amplification (Table 1 and Fig. 1C and D). Chelex does not eliminate all PCR inhibitors, but an easy way to remove them is to dilute

Table 1
Microbial counts and DNA extraction efficiency from eggshell surfaces.

Sample	Avian species	Microbial counts[a] (Log CFU/clutch)				Eggs/clutch	16S PCR DNA dilution			16S PCR (plus BSA) DNA dilution		
		TSA	HK	KF	VJ		0	1:10	1:100	0	1:10	1:100
Cast. Quem.2	*Upupa epops*	7.151	7.084	NG	4.623	4	–	–	+	–	+	+
H789.1	*Upupa epops*	7.955	7.532	5.441	NG	8	+	+	+	+	+	+
C.44.1	*Upupa epops*	5.155	4.812	3.429	2.193	7	+	+	+	+	+	+
H851	*Upupa epops*	8.121	7.924	7.096	NG	7	+	+	+	+	+	+
H.HV14.1	*Upupa epops*	8.121	7.857	7.136	3.183	7	+	+	+	+	+	+
TEJ 25.1	*Parus major*	6.158	6.960	1.857	1.380	8	+	+	+	+	+	+
CA51.2	*Parus major*	5.778	4.477	3.158	NG	10	+	+	–	+	+	+
C18.1	*Parus major*	3.778	2.742	1.079	NG	7	+	+	–	+	+	–
CA61.1	*Parus major*	2.121	1.857	NG	NG	4	–	+	–	–	+	–
CA51.1	*Parus major*	7.885	7.635	3.799	NG	10	+	+	+	+	+	+
BEC 15.1	*Athene noctua*	3.778	2.742	1.079	NG	3	+	+	+	+	+	+
769.1	*Athene noctua*	8.310	8.193	6.692	1.079	3	+	+	+	–	+	+
133.1	*Athene noctua*	7.376	5.426	3.320	5.523	3	+	+	+	–	+	+
842.1	*Athene noctua*	7.730	6.225	5.792	NG	5	+	+	+	+	+	+
55.1	*Athene noctua*	7.955	8.121	3.788	NG	5	+	+	+	+	+	+
P25.2	*Sturnus unicolor*	4.769	3.294	1.778	1.380	5	+	+	–	+	+	+
HUE 1.1	*Sturnus unicolor*	8.121	7.677	8.598	NG	6	+	+	+	–	+	+
HUE 31.1	*Sturnus unicolor*	5.487	5.317	2.732	1.079	4	–	+	+	–	+	–
HUE 27.1	*Sturnus unicolor*	7.459	7.196	4.334	1.380	6	+	+	+	+	+	+
HUE 39.1	*Sturnus unicolor*	9.576	8.401	3.291	NG	5	+	+	+	+	+	+
N29.1	*Pica pica*	7.009	6.659	NG	NG	8	–	+	+	–	+	+
N7.1	*Pica pica*	5.304	2.905	NG	NG	7	–	+	–	–	+	–
N32.1	*Pica pica*	8.494	7.694	3.412	1.079	6	+	+	+	+	+	+
N3.1	*Pica pica*	8.611	8.477	NG	NG	7	+	+	+	+	+	+
N32.1c	*Clamator glandarius*	4.225	2.494	NG	NG	2	–	+	+	–	+	–

Note. NG, no growth.
[a] TSA for total aerobic mesophilic bacteria, HK for enterobacteria, KF for enterococci, and VJ for staphylococci.

the DNA solution 10- or 100-fold [5,12], so we used diluted DNA as template, getting the best results in DNA diluted 1:10 (24 positives). Moreover, because of the risk of high protein content in the DNA extracted due to the simplicity of the method, we repeated the 16S amplification and included bovine serum albumin (BSA, 200 ng/μl) in the PCR reaction mixture to protect the Taq polymerase from the possible presence of protease and PCR inhibitors [5,13]. In this way, we could amplify the 16S gene of the microbial communities of all the samples studied using the isolated DNA diluted 1:10 (Table 1 and Fig. 1C). A TTGE analysis, a measure of microbial diversity from eggshells, is presented to test this method (Fig. 1D). When different kits or traditional techniques were used to purify DNA from these communities, only 20% of samples provided a PCR product for 16S ribosomal DNA (results not shown).

In conclusion, we have successfully analyzed eggshell microbial communities from six avian species with a high variation in their microbiological levels using this simple, low-cost, and rapid method, which ideally can be applied to a high number of samples.

Acknowledgments

This work was supported by the Junta de Andalucía (RNM 345) and the Spanish Ministerio de Educacion y Ciencia as well as the European founds FEDER (CGL2007-61251). Juan Manuel Peralta-Sánchez worked with a grant from the Junta de Andalucia (RNM 345).

References

[1] J. Bruce, E.M. Drysdale, Trans-shell transmission, in: R.G. Board, R. Fuller (Eds.), Microbiology of the Avian Egg, Chapman & Hall, London, 1994, pp. 63–91.
[2] M.S. Rappé, S.J. Giovannoni, The uncultured microbial majority, Annu. Rev. Microbiol. 57 (2003) 369–394.
[3] K.H. Schleifer, Microbial diversity: facts, problems, and prospects, Syst. Appl. Microbiol. 27 (2004) 3–9.
[4] M.D. Shawkey, M.K. Firestone, E.L. Brodie, S.R. Beissinger, Avian incubation inhibits growth and diversification of bacterial assemblages on eggs, PLoS ONE 4 (2009) e4522.
[5] P.M. Scheu, K. Berghof, U. Stahl, Detection of pathogenic and spoilage microorganisms in food with the polymerase chain reaction, Food Microbiol. 15 (1998) 13–31.
[6] C.W. Huang, M.C. Huang, A simple and rapid PCR-based method for ostrich sexing using micro amounts of DNA, Reprod. Domest. Anim. 43 (2008) 643–646.
[7] O. Tomasek, O. Kubicek, V. Tukac, Comparison of three template preparation methods for routine detection of beak and feather disease virus and avian polyomavirus with single and nested polymerase chain reaction in clinical specimens, Avian Pathol. 37 (2008) 145–149.
[8] X. de Lamballerie, C. Zandotti, C. Vignoli, C. BolLlet, P. de Micco, A one-step microbial DNA extraction method using "Chelex 100" suitable for gene amplification, Res. Microbiol. 143 (1992) 785–790.
[9] G. Giraffa, L. Rossetti, E. Neviani, An evaluation of chelex-based DNA purification protocols for the typing of lactic acid bacteria, J. Microbiol. Methods 42 (2000) 175–184.
[10] J.C. Ogier, O. Son, A. Gruss, P. Tailliez, A. Delacroix-Buchet, Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis, Appl. Environ. Microbiol. 68 (2002) 3691–3701.
[11] M.I. Cook, S.R. Beissinger, G.A. Toranzos, R.A. Rodriguez, W.J. Arendt, Trans-shell infection by pathogenic micro-organisms reduces the shelf life of non-incubated bird's eggs: a constraint on the onset of incubation?, Proc R. Soc. Lond. B 270 (2003) 2233–2240.
[12] S. Ishii, T.E. Loynachan, Rapid and reliable DNA extraction techniques from trypan-blue-stained mycorrhizal roots: comparison of two methods, Mycorrhiza 14 (2004) 271–275.
[13] P. Radström, R. Knutsson, P. Wolffs, M. Lövenklev, C. Löfström, Pre-PCR processing, Mol. Biotechnol. 26 (2004) 133–146.